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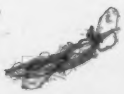
Regulation of Intestinal Proglucagon Expression and GLP-1 Secretion by Diet in Normal and Diabetic States

by

Raylene Alison Reimer



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **REGULATION OF INTESTINAL PROGLUCAGON EXPRESSION AND GLP-1 SECRETION BY DIET IN NORMAL AND DIABETIC STATES** submitted by Raylene Alison Reimer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition and Metabolism.

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Abstract

Intestinal delivery of nutrients and hormones to systemic circulation may significantly affect metabolism and risk and/or management of chronic diseases. The objective of this work was to characterize the effect of diet on the expression of intestinal proglucagon mRNA and GLP-1 secretion in normal and diabetic states. Fermentable dietary fiber increased ileal proglucagon mRNA and insulin secretion in normal adult rats. Adult diabetic rats responded differently to fermentable fiber than normal rats. Ileal and colonic proglucagon mRNA and secretion of GLP-1 are significantly affected by diet (rhubarb versus cellulose), whereas SGLT-1 and GLUT2 mRNA and plasma glucose and insulin were also responsive to food intake. Weaning to chow diets results in a higher incidence of diabetes in BBdp rats than weaning onto semi-purified diets. Consumption of diets high in fermentable fiber between 21 and 30 days of age increased proglucagon and intestinal glucose transporter mRNA abundance in BBdp rats compared to BBn rats. Secretion of GLP-1, a potent insulin secretagogue, was higher in BBdp rats at 30 days of age compared to BBn rats. At 30 days of age, BBdp rats fed chow had greater small intestine and colon weight as a proportion of body weight than BBdp rats fed casein or soy diets compared to their BBn counterparts. In BBn rats chow resulted in greater intestinal growth and abundance of SGLT-1 and GLUT5 mRNA. In the BBdp rat, SGLT-1 and GLUT5 mRNA and intestinal and body weight were not altered with diet. Characterization of the actions of GLP-1 on skeletal muscle showed that prolonged exposure of epitrochlearis muscle to GLP-1 *in vitro* inhibits insulin-stimulated glucose uptake. The antagonist and homologue to GLP-1, exendin 9-39, also inhibited insulin-stimulated glucose uptake in epitrochlearis muscle *in vitro*. In summary, these studies

have examined several molecular mechanisms governing intestinal adaptation in response to diet and specifically dietary fiber. Alterations in various markers of glucose absorption and disposal have been examined. Future work will ultimately enable nutrition experts to design diets, whether for infancy or adulthood, which will optimize intestinal function and promote glucose homeostasis.

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LIST OF ABBREVIATIONS

ATP = adenosine triphosphate

BBdp = Biobreeding diabetes prone

BBM = brush border membrane

BBn = Biobreeding control

BLM = basolateral membrane

C = degrees Celsius

cAMP = cyclic adenosine monophosphate

cDNA = complementary deoxynucleic acid

EGF = epidermal growth factor

GIP = glucose dependent insulinotropic polypeptide

GLP-1 = gluagon-like peptide-1

GLUT2 = sodium independent glucose transporter

GLUT5 = sodium independent glucose transporter

GRPP = Glicentin-related pancreatic polypeptide

IDDM = insulin dependent diabetes mellitus

IP-2 = intervening peptide-2

Km = Michaelis constant

MPF = major proglucagon fragment

mRNA = messenger ribonucleic acid

MSBR = massive small bowel resection

NIDDM = non-insulin dependent diabetes mellβitus

PACAP = Pituitary adenylate cyclase activating peptide

RT-PCR = reverse transcriptase

SAS = statistical analysis system

SCFA = short chain fatty acid

SEM = standard error of the mean

SGLT-1 = sodium dependent D-glucose cotransporter

SP = Semi-purified diet

TPN = total parenteral nutrition

VIP = vasoactive intestinal peptide

Vmax = maximal transport rate

CHAPTER 1

LITERATURE REVIEW

1 INTRODUCTION

It is well accepted that dietary fiber is able to improve glycemic control in diabetes. Metabolic response to a glucose challenge in humans has shown that high fiber supplemented diets flatten the glucose curve and can decrease fasting insulin and glucose levels (Jenkins et al, 1976; Miranda & Horwitz, 1978; Pastors et al, 1991; Fukagawa et al, 1990). High fiber diets have also been reported to improve insulin sensitivity of the body's peripheral tissues and thereby reduce insulin dosage (Anderson, 1986; Fukagawa, 1990). The precise mechanisms whereby dietary fiber confers benefits to subjects with diabetes remains to be elucidated.

While the physical form of dietary fiber i.e. the ability to form a viscous gel explains the slowing of nutrient absorption and the benefits of fiber seen when fiber is present in a meal the precise mechanisms responsible for improvements seen when fiber is not physically present in the intestine such as with an oral glucose load after an overnight fast remain unclear. Only recently has it been suggested that effects on glycemia and insulin secretion may be mediated via gastrointestinal hormones.

Glucagon-like peptide-1 is a gastrointestinal hormone which acts as a potent insulin secretagogue (Holst, 1994). Expression of the precursor, proglucagon, is known to change with weaning, age, intestinal resection and in certain disease states such as diabetes mellitus (Bloom & Polak, 1982; Taylor et al, 1990). Adaptation in the gastrointestinal tract with long-term ingestion of dietary fiber may alter the expression and secretion of proglucagon and GLP-1 respectively and play a role in improving glucose homeostasis.

2 INCRETINS

2.1 DEFINITION

Oral glucose augments insulin secretion to a greater extent than intravenous glucose infusion in normal subjects (Perley & Kipnis, 1967; Nauck et al, 1986). This increase in secretion of insulin after oral glucose is ascribed to the action of specific gastrointestinal hormones. Glucose-dependent insulinotropic polypeptide (GIP) released from the upper intestine (Nauck et al, 1989) and glucagon-like peptide (GLP)-1, released from the lower intestine (Orskov et al, 1989; Varndell et al, 1985) are insulinotropic hormones released in response to oral nutrients. Dose response analysis of the insulinotropic activity of GIP and GLP-1 indicate that lower plasma concentrations of GLP-1 (~10-100 pmol/l) are necessary to enhance insulin secretion (Holst et al, 1987; Mojsov et al, 1987; Weir et al, 1989) than is known for GIP especially at elevated glucose concentrations (Nauck et al, 1989; Creutzfeldt, 1979). The precise nature of the individual and interactive role of these two hormones in glucose homeostasis in normal and diabetic states is of great interest but as yet unclear.

2.2 ACTIONS OF INCRETINS IN NORMAL AND DIABETIC STATES

In NIDDM, the incretin effect is reduced or lost (Nauck et al, 1986; Tronier et al, 1985). This does not, however, result from a decreased secretion of GLP-1 or GIP because normal or even elevated circulating levels of these hormones are found in NIDDM patients (Nauck et al, 1986; Nauck et al, 1993; Orskov et al, 1991). For example, fasting GLP-1 levels in healthy volunteers were 37±3 pmol/l and rose approximately twofold after an oral glucose load peaking at 30 minutes. Fasting GLP-1 levels in patients with NIDDM were significantly higher (47±7 pmol/l) rising to ~100 pmol/l after oral glucose, similarly peaking at 30 minutes (Orskov et al, 1991).

The decreased incretin effect seen in diabetics may be caused by a deficient action of these hormones at the β-cell level, either as a result of decreased receptor number or because of defects in intracellular signal transduction. Both GIP and GLP-1 have specific β-cell receptors that are coupled to production of cAMP and activation of cAMP-dependent protein kinase. Elevation in intracellular cAMP levels is required to mediate the glucoincretin effect of these hormones (Thorens & Waeber, 1993). In addition, circulating glucoincretins maintain basal levels of cAMP which are necessary to keep the β-cells in a glucose-competent state. Interactions between glucoincretin signalling and glucose-induced insulin secretion may result from phosphorylation of key elements of the glucose signalling pathway by cAMP-dependent protein kinase. These include the ATP-dependent K^+ channel, the Ca^{2+} channel or elements of the secretory machinery itself (Thorens & Waeber, 1993).

Holz et al (1993) defined glucose competence as the glucose sensitivity conferred to glucose resistant β-cells by GLP-1. By acting synergistically with glucose, GLP-1 inhibits metabolically regulated potassium channels that are also targeted for inhibition by sulphonylurea drugs. Glucose competence allows membrane depolarization, the generation of action potentials, and Ca^{2+} influx, events that are known to trigger insulin secretion.

Recent studies using pharmacological doses have shown that infusion of GLP-1 but not GIP could ameliorate glucose levels by stimulating insulin secretion in NIDDM patients (Gutniak et al, 1992; Nathan et al, 1992; Nauck et al, 1993). At equimolar concentrations and infusion rates, GLP-1 is more potent than GIP in stimulating the β-cell to secrete insulin (Andersen et al, 1990; Shima et al, 1988). With GIP, insulin secretion in NIDDM patients was significantly lower (by 54%) than in normal subjects. With GLP-1(7-36)amide, NIDDM patients reached 71% of the increment in C-peptide of normal subjects.

GLP-1(7-36)amide is also able to slightly but significantly lower pancreatic glucagon in normal subjects and much more prominently in NIDDM. Exogenous GIP was without major effect on pancreatic glucagon concentrations in both subject groups. Data suggests that GLP-1 decreases glucagon secretion indirectly via the stimulation of the release of insulin and probably somatostatin (Suzuki et al, 1989; Fehmann & Habener, 1991a). These hormones in turn have a suppressive effect on the glucagon producing alpha cells.

The relative quantities and actions of GIP and GLP-1 in NIDDM differ from normal subjects. In normal subjects an additive insulinotropic effect is seen with the two peptides with GIP making a quantitatively more important contribution (Nauck et al, 1993). The reason for the disparity between normal and diabetic response is unknown.

The potential use of GLP-1 as the newest antidiabetogenic agent is supported by research thus far. The reasons that GLP-1 is such an attractive therapeutic agent for the treatment of NIDDM are numerous and the potential for diet to play a role in modulating the endogenous secretion of GLP-1 is of great interest. In summary the advantages of GLP-1 use include: a) the preservation of the insulinotropic activity of GLP-1 in NIDDM (Nauck et al, 1993); b) GLP-1 also has glucagon lowering effects (Nauck et al, 1993); c) inhibition of gastric emptying rates (Schjoldager et al, 1990; Gutniak et al, 1992; Willms et al, 1996) thereby lowering the need for a rapid insulin secretory response; d) the postulated glucose dependency of the insulinotropic actions of GLP-1(7-37) theoretically limit the risk of hypoglycemia often seen with subcutaneously administered insulin or oral sulphonylureas.

As revealed by the preceding discussion, GLP-1 has potential for therapeutic treatment of NIDDM. Examination of factors modulating the expression and secretion of this hormone will broaden our understanding of the role this incretin plays in normal physiological and diabetic states. Processing of the proglucagon gene has several interesting features and warrants further discussion.

3 GLUCAGON-LIKE PEPTIDES

3.1 STRUCTURE

Proglucagon is a 160 amino acid precursor encoded by a single gene (Mojsov et al, 1986). In addition to glicentin and glucagon, the precursor contains the sequence of two glucagon-like peptides, GLP-1 and GLP-2, separated by an intervening peptide (IP-2) (Heinrich et al, 1984; Bell et al, 1983; Lopez et al, 1983). Processing of proglucagon differs in the pancreatic alpha cells and intestinal L cells. Predominant products in the pancreas are glucagon, GLP-1 and the major proglucagon fragment (MPF) and predominant products in the intestine are glicentin, GLP-1, GLP-2 and IP-2 (Mojsov et al, 1986; George et al, 1985) **(Figure 1.1)**.

3.2 POST-TRANSLATIONAL PROCESSING

Tissue specific post-translational processing occurring in the pancreas and intestine allows biologically active peptides to be generated from larger inactive proteins. Endoproteolytic cleavage generally occurs at sites containing 1-4 basic amino acids (lysine, arginine). Both GLP-1 and GLP-2 are flanked at their amino and carboxy termini by basic residue pairs (Mojsov et al, 1990). GLP-1 is also the substrate for a second proteolytic enzyme in the gut with cleavage occurring at a single arginine residue in the amino terminal region liberating the 31 amino acid peptide GLP-1(7-37) or GLP-1(7-36)amide. These truncated peptides are the predominant molecular form of GLP-1 in the intestine with the amide form accounting for ~80% and the glycine extended form accounting for ~20% of GLP-1 immunoreactivity in humans (Orskov et al, 1994). In rats, this fraction is approximately 2/3 and 1/3 respectively (Mojsov et al, 1990).

3.3 Immunoreactivity in Plasma

GLP-1 immunoreactivity in plasma is found principally in the mature bioactive GLP-1 peptides derived from the intestine and major proglucagon fragment, the end product of pancreatic processing cosecreted with glucagon from the alpha cells into the circulation. During intravenous infusion of arginine, a potent direct secretagogue of the pancreatic alpha cell, GLP-1 immunoreactivity is contained within a high molecular weight protein, most likely representing the biologically inert MPF. Conversely, after a meal nearly all of the GLP-1 immunoreactive material derives from the intestine and consists of the bioactive GLP-1 hormone isoforms, GLP-1(7-37) and GLP-1(7-36)amide (Orskov et al, 1991). Recent development of an antiserum specific for GLP-1 has enabled the determination of GLP-1 in plasma of human subjects. In healthy volunteers the fasting GLP-1 levels are 30-40 pmol/l and rise about twofold after an oral glucose load with a peak level occurring at 30 minutes and declining to basal values after 180 minutes (Orskov et al, 1991). As mentioned previously, basal levels as well as GLP-1 immunoreactivity up to 60 minutes after oral glucose and intravenous arginine administration are significantly higher in NIDDM compared to normal subjects.

3.4 Biological Actions

Convincing evidence exists suggesting that the truncated forms of GLP-1, GLP-1(7-36)amide and /or GLP-1(7-37) are physiologically important incretins. Studies in vitro have shown that GLP-1(7-37) concentrations as low as 50 pmol/l will stimulate insulin release from isolated perfused rat pancreas (Mojsov et al, 1987). In all experimental systems thus far investigated, GLP-1 stimulates insulin release only in the presence of elevated levels of glucose. In humans, the infusion of synthetic GLP-1 in physiologic amounts results in a prompt increase of insulin secretion (Kreymann et al, 1987; Nathan et al, 1992).

As opposed to sulfonylurea drugs which merely increase insulin secretion, GLP-1 is able to stimulate proinsulin biosynthesis. In the rat insulinoma cell line RIN 1046-38, GLP-1 increased levels of proinsulin mRNA (Drucker et al, 1987). Other studies at the molecular level confirm the role of GLP-1 as an insulinotopic hormone (Philippe & Missotten, 1990). In addition to its potent insulinotropic actions the truncated GLP-1(7-36)amide is a unique gastrointestinal hormone in that it is also glucagonostatic. Significant inhibitory effects on glucagon secretion have been demonstrated at levels of GLP-1(7-36)amide as low as 0.25 nM (Komatsu et al, 1989; Kreymann et al, 1987; Orskov et al, 1988). GLP-1 appears to participate in an enteroinsular control of pancreatic endocrine secretion.

As well as stimulating insulin secretion, GLP-1 also acts on the insulin-independent fraction of glucose disposal. D'Allessio et al (1995) demonstrated that GLP-1 was able to significantly increase glucose effectiveness. This insulin-independent glucose disposition accounts for an estimated 40-60% of glucose disposition after oral glucose (Henriksen et al, 1994). Only recently has the use of the minimal model to estimate changes in glucose effectiveness in subjects with normal insulin secretory function been challenged and therefore the precise role of GLP-1 as determined with this model is yet unresolved.

3.5 Regulation of Proglucagon

3.5.1 Regulation of expression

The proglucagon gene is known to adapt under various conditions including intestinal resection and age. Intestinal proglucagon mRNA can be detected as early as day 11 of gestation which suggests independent regulation of differentiation in pancreatic alpha cells versus intestinal L cells. Intestinal proglucagon synthesis in developing rats precedes that of gastrin (Larsson et al, 1974), somatostatin (Alumets et al, 1977), secretin (Larsson et al, 1977), vasoactive intestinal peptide (Laburthe, 1977) and glucose-

dependent insulinotropic polypeptide (Gespach et al, 1979) implying a role for GLP in intestinal development and growth. Intestinal content of GLP-1(7-36)amide increased 100 times between fetal levels and 30 days with greatest increment seen at weaning (Kreymann, 1991). Introduction of solid food and specifically dietary fiber may play a role in modulating the levels of proglucagon at this ontogenic stage.

Upregulation of proglucagon mRNA following massive small bowel resection (MSBR) is a well established phenomenom (Taylor et al, 1990). Similar to the early postnatal period, the small bowel undergoes an adaptive increase in length, weight, and crypt cell hyperplasia (Herbst & Sunshine, 1969). Although evidence for the role of enteroglucagon (glicentin and oxyntomodulin) in intestinal adaptation is indirect it is commonly considered as the enterotrophic humoral factor involved in the post MSBR changes (Rountree et al, 1992). In addition to increased proglucagon gene expression, levels of enteroglucagon in the blood and bowel are elevated as well (Rountree et al, 1992). In this model it has been established that there is an increase in the amount of proglucagon mRNA in a given L cell rather that an increase in the number of L cells (Fuller et al, 1993).

Tappenden et al (1996) have also demonstrated that proglucagon mRNA is elevated in rats administered total parenteral nutrition (TPN) supplemented with SCFA as compared to those receiving an isocaloric/isonitrogenous TPN solution. These increases are present at 3 and 7 days following massive small bowel resection (MSBR) but have been detected as early as 6 hours following SCFA supplementation after surgery (Tappenden et al, unpublished data).

3.5.2 Regulation of Secretion of GLP-1

Development of *in vitro* models with intestinal L cell make it possible to study the factors regulating the synthesis and secretion of intestinal proglucagon-derived peptides.

Intracellular control of synthesis and secretion of intestinal proglucagon peptides are stimulated by receptor-independent activation of the protein kinase A pathway. Only secretion, however, is stimulated by activation of the protein kinase C pathway (Drucker et al, 1989; Brubaker et al, 1988).

Secretion of GLP-1 immunoreactivity has been reported to be modulated by luminal glucose and the neuropeptide gastrin-releasing peptide in isolated perfused ileum (Orskov et al, 1986; Namba et al, 1990). The broader spectrum glucagon-like immunoreactivity which includes glicentin and oxyntomodulin co-released with GLP-1 has been shown both *in vivo* and *in vitro* to be regulated by intraluminal nutrients (Roberge & Brubaker, 1991; Brubaker & Vranic, 1987) as well as hormones and neurotransmitters, including somatostatin, GIP, gastrin-releasing peptide and cholinergic agonists (Brubaker, 1991; Buchan et al, 1987).

Early studies addressing the effects of different nutrients on GLP-1 secretion display discrepancies in reported levels of GLP-1 released (Kreymann et al, 1987; Hirota et al, 1990; Orskov & Holst, 1987) largely due to the varying cross-reactivity with extended forms of pancreatic GLP-1 and problems associated with different plasma extraction techniques. Using a double-antibody disequilibrium assay with 200 ul of unextracted plasma, Elliot et al (1993) measured immunoreactive GLP-1 release in humans in response to several nutrients and test meals. Isoenergetic carbohydrate, fat or protein meals were able to significantly elevate immunoreactive GLP-1. This increase was small and transient and only the carbohydrate meal altered plasma glucose and insulin levels. The simple carbohydrate, glucose, caused a significant rise in GLP-1 levels whereas complex carbohydrates, rice and barley meals, did not significantly increase circulating GLP-1 levels. Over a 24 hour period which included main meals and snacks, GLP-1 rose following consumption of each main meal, but unlike glucose and insulin, remained elevated throughout the daytime, only reaching basal levels after an overnight fast. The continuously elevated daytime levels of GLP-1 suggest that most people are absorbing

nutrients, particularly fat, from the gut for approximately 18 out of 24 hours. Previous work has shown that both GIP and GLP-1 have anabolic effects on lipid metabolism by stimulating fatty acid synthesis and increasing lipoprotein lipase activity (Oben et al, 1991 a, b). The observation that glucose and insulin levels decline to basal after a meal whereas GIP and GLP-1 remain elevated suggests a possible role of GLP-1 in the metabolism of the fat component of a mixed meal.

It has been suggested that GLP-1 secretion is stimulated by glucose absorption and that a minimum rate of glucose absorption is necessary for an appreciable increase in GLP-1 concentrations to be observed. Recent work by Plaisancie et al (1995) however demonstrated that perfusion of an isolated colonic loop with pectin was able to evoke a prompt and sustained increase in secretion of GLP-1. Perfusions with cellulose and gum arabic were without effect on GLP-1 secretion. In addition, only one of the bile acids, hyodeoxycholate, was able to significantly stimulate GLP-1 secretion. These findings suggest that absorption of glucose may not be a prerequisite for the stimulation of GLP-1 secretion from the colon.

3.6 GLP-1 Receptor

3.6.1 Tissue Distribution

The receptor for GLP-1 has recently been cloned and the availability of the complementary DNA has provided a valuable tool for quantifying mRNA for the GLP-1 receptor (Thorens, 1992; Graziano et al, 1993; Dillon et al, 1993). Reports on the tissue distribution of GLP-1 receptor mRNA have been numerous and conflicting (Thorens, 1992; Wei & Mojsov, 1995; Campos et al, 1994; Egan et al, 1994). Using the most sensitive methods of detection, the RNase protection assay and RT-PCR, Bullock et al, (1996) were unable to detect any GLP-1 receptor mRNA in rat liver, adipose tissue or gastrocnemius muscle. The authors suggest that the reported effects of GLP-1 in these

tissues may not occur through an interaction with the pancreatic form of the GLP-1 receptor. Binding may be to other GLP-1 related receptors or the structurally similar VIP or PACAP receptors.

Binding studies, however, have described GLP-1 receptors in several extrapancreatic tissues including brain (Uttenthal et al, 1992; Shimizu et al, 1987; Kanse et al, 1988), lung (Kanse et al, 1988; Richter et al, 1990), and gastric glands of the stomach (Uttenthal & Blazquez, 1990). Three key tissues involved in glucose regulation in the body, ie. liver, adipose and muscle, have also demonstrated binding with GLP-1.

Evidence for the role of GLP-1 at these tissues is only now emerging and remains controversial. Recently the presence and characteristics of GLP-1 receptors in plasma membranes in rat liver has been demonstrated (Villaneuva-Penacarrillo et al, 1995). In isolated rat hepatocytes, physiological concentrations of GLP-1(7-36) amide exerted potent glycogenic actions (Valverde et al, 1994). Other reports, however, have not been able to demonstrate any effect of GLP-1 on cAMP, Ca^{2+} levels or carbohydrate metabolism in isolated hepatocytes or glycogenolysis/ketogenesis in perfused rat liver (Blackmore et al, 1991; Ghiglione et al, 1985; Murayama et al, 1990).

GLP-1 has also been shown to bind with high affinity to isolated rat adipocytes. At this site the actions of GLP-1 include the production of cAMP (Valverde et al, 1993), stimulation of lipogenesis (Oben et al, 1991) or lipolysis (Ruiz-Grande et al, 1992).

Finally the presence of GLP-1 receptors on skeletal muscle (Delgado et al, 1995; Villaneuva-Penacarrillo et al, 1995), a major glucose utilizing organ suggests that in this tissue as well as in the liver and adipose, GLP-1 may play an important role in regulation of whole body glucose homeostasis. Reports on the actions of GLP-1 in the muscle are yet controversial with Villaneuva-Penacarrillo et al (1994) describing potent glycogenic effects while Furnsinn et al (1995) were unable to reproduce those findings.

3.6.2 ANTAGONIST/AGONIST TO GLP-1 RECEPTOR

Until recently the absence of a potent inhibitor of GLP-1 limited the determination of the precise role of this peptide in glucose homeostasis. The discovery of exendin-(9-39) as a specific GLP-1 receptor antagonist has provided a useful tool to assess the importance of GLP-1 in the enteroinsular axis. Several studies indicate that two peptides isolated from the venom of the lizard *Heloderma suspectum*, exendin-4 and exendin-(9-39), are an avid agonist and antagonist respectively of the GLP-1 receptor (Goke et al, 1993; Raufman et al, 1992; Thorens et al, 1993). Goke et al (1993) demonstrated that exendin-4 mimics the actions of GLP-1 by stimulating an increase in cellular cAMP and enhancing glucose-induced insulin release from pancreatic beta-cells. Exendin-(9-39) amide is able to inhibit these biological actions. Interestingly, the exendins share 53% sequence homology with GLP-1(7-36) amide.

Two groups have employed the use of exendin-(9-39) to determine the physiological role of GLP-1. Wang et al (1995) demonstrated in anaesthetized rats that exendin-(9-39) amide inhibited GLP-1 induced insulin secretion in a dose dependent manner. In conditioned conscious rats receiving a standard meal, postprandial insulin release was reduced by 48% with subcutaneous injection of exendin-(9-39) amide. Similarly, Kolligs et al (1995) demonstrated that exendin-(9-39) amide abolished the insulin-stimulatory effect of GLP-1 and the GLP-1 agonist, exendin-4. Intravenous injection of the antagonist just prior to enteral glucose infusion greatly reduced the insulin secretory response and elevated blood glucose. Taken together these studies provide strong evidence for the role of GLP-1 as a hormonal mediator in the enteroinsular axis.

3.6.3 DESENSITIZATION OF THE GLP-1 RECEPTOR

As stated earlier, the incretin activity of GIP is greatly diminished in patients with NIDDM whereas that of GLP-1 remains normal (Elahi et al, 1994; Nauck et al, 1993).

Tseng et al (1996) recently demonstrated that GIP gene expression is enhanced in diabetic animals and that elevated serum GIP induces chronic desensitization of the GIP receptor in vivo. Because elevated levels of GLP-1 throughout the day may be needed to achieve optimal blood glucose control, desensitization of the GLP-1 receptor must also be considered in the therapeutic use of GLP-1(7-37) in NIDDM. Indeed rapid and reversible homologous desensitization of responses to GLP-1(7-37) has been demonstrated in rat insulinoma HIT-T15 cells (Fehmann & Habener, 1991).

More recently, Gronau & Brubaker (1995) demonstrated that the GLP-1(7-37)/(7-36) amide receptor in normal islets may undergo homologous desensitization as well with chronic, 24 h exposure to GLP-1. The loss of GLP-1 bioactivity appeared to be related to a dissociation of the GLP-1 receptor from adenylyl cyclase and not to a dissociation of cAMP from stimulation of hormone secretion. Additionally, Gromada et al (1996) determined that desensitization of the GLP-1 receptor does not result from the activation of protein kinase A or Ca^{2+}-dependent kinases/phosphatases. Protein kinase C-dependent mechanisms appear to mediate in part the desensitization. From a physiological standpoint, desensitization may act in protecting against hypoglycemia in excessive GLP-1 induced insulin secretory states.

Kawai & Ohashi (1993) reported that long term (30 day) administration of GLP-1(7-36) amide had no beneficial effect on oral glucose tolerance in normal rats and only a tendency for increased insulin and decreased glucose before and after oral glucose in diabetic rats. Animals in a study by Hargrove et al (1995), however, had not become completely desensitized to GLP-1(7-37). In this regard, more detailed long-term dosing studies are needed to fully assess the insulinotropic activity of GLP-1(7-37)/(7-36) amide in vivo.

4 *Intestinal Adaptation in Diabetes Mellitus*

The small intestine is a dynamic organ possessing a remarkable ability to alter nutrient absorptive capacity in response to a number of physiological and pathological conditions. These include developmental age, pregnancy and lactation, surgical resection of the intestine, disease states such as diabetes, thyrotoxicosis and starvation and alterations in type and quantity of diet (Philpott et al, 1992).

4.1 Adaptation in Streptozotocin Induced Diabetes

Adaptation can occur through non-specific mechanisms including morphological change as well as through more specific mechanisms centered at the enterocyte in the small intestine (Thomson, 1981; Fedorak et al, 1990; Anderson, 1974; Chang et al, 1986; Schedl and Wilson, 1971; Miller et al, 1977). Streptozotocin-induced diabetes mellitus in the rat is a useful model to study the characteristic increase in the intestinal uptake of nutrients observed in the diabetic state.

4.1.1 non-specific Adaptation

Small intestinal growth and function are altered in the rat after the induction of diabetes by alloxan or streptozotocin. Despite catabolism of other tissues, intestinal mass and length increase in diabetic compared to control rats (Ferraris et al, 1993; Pillion et al, 1988; Schedl and Wilson, 1971; Jervis and Levin, 1966). These changes are likely a compensatory mechanism to increase nutrient absorption in a perceived state of tissue starvation and are primarily due to increased DNA synthesis and crypt cell production rates (Bloom and Polak, 1982; Miazza et al, 1985). Insulin treatment of diabetic rats lowers crypt production toward normal, suggesting that increased DNA synthesis is a result of the diabetic state and is reversed by subsequent control of the diabetes (Miller et al, 1977).

In the past researchers believed that a direct correlation existed between the increased quantity of food consumed by diabetic rats and the hyperplasia of the small intestine. Investigations since that time have shown similar hyperplastic intestinal changes in diabetic rats fed diets isocaloric with those of controls, suggesting that diabetes causes organ specific changes in the gastrointestinal tract in the absence of hyperphagia (Granneman and Stricker, 1984; Miller et al, 1977).

In addition to an increase in the length of the small intestine and increase in the number of villi other mechanisms may affect glucose absorption in a non-specific way. Decreases in the thickness of the unstirred water layer, increased concentration of hydrolases and a general increase in membrane permeability may all contribute to a non-specific increase in glucose uptake from the small intestine (Younoszai & Schedl, 1972; Thomson, 1983; Hotke et al, 1985). Much of the fine tuning of nutrient absorption, however, takes place at the level of the enterocyte itself and includes more specific mechanisms of upregulation.

4.1.2 SPECIFIC ADAPTATION

Specific mechanisms which regulate the absorption of glucose from the small intestine include alterations in uptake via tight junctions (paracellular pathway) and the enterocyte (transcellular pathway) (Ballard et al, 1995; Karasov & Diamond, 1983). The potential sites of regulation are depicted in **Figure 1.2**.

The transcellular pathway involves specific carriers on the brush border and basolateral membrane of the enterocyte. Transport of glucose and galactose across the BBM of the intestinal enterocyte is by the sodium-dependent glucose transporter (SGLT-1) (Hediger et al, 1987). GLUT5 is now recognized as the intestinal BBM transporter for fructose (Burant & Bell, 1992). The exit or efflux of hexoses from the enterocytes occurs via the facilitative glucose transporter gene family (GLUT2) (Thorens et al, 1990). Only recently

has GLUT5 been localized to the BLM as well and this suggests that it participates in the transfer of fructose across the basal membrane of the enterocyte as well (Blakemore et al, 1995).

Several factors have been shown to regulate uptake via membrane associated carriers. Studies examining nutrient absorption depict an enhanced rate of glucose absorption with increases in dietary carbohydrate (Ferraris and Diamond, 1986; Diamond and Karasov, 1984). This increase in glucose transport was caused by an increase in the maximal rate of glucose transport (Vmax) while the carriers' affinity for glucose remained unchanged.

Enhancement of BBM glucose absorption is also seen in streptozotocin induced diabetes mellitus (Dudeja et al, 1990; Fedorak et al, 1987, 1989; Thomson, 1981). The increase in glucose flux across the enterocyte in streptozotocin-induced diabetes is due to the increased expression of SGLT-1 (Miyamoto et al, 1991; Burant et al, 1994). In addition, Burant et al (1994) have also shown that to a lesser extent expression of GLUT5 is also increased in experimental diabetes. In addition to increasing the levels of transporter mRNA normally expressed in enterocytes, diabetes causes a premature expression of the genes in the more immature cells of the lower villus and crypt (Burant et al, 1994).

Suggestions that mechanisms at the BLM were involved in the rapid upregulation of glucose transport induced by hyperglycemia first emerged in 1981 (Csaky & Fisher, 1981). Since that time others have confirmed that hyperglycemia results in a rapid increase in glucose transport across the BLM (Cheeseman & Maenz, 1989; Karasov & Diamond, 1987).

Further characterization by Cheeseman and Maenz (1989) suggests that initially hyperglycemia results in a stimulation of carrier activity followed by recruitment of new BLM carriers. These new carriers may, however, differ from those found at the basal state by increasing their affinity for glucose or causing more rapid movement of glucose

out of the cell. The upregulation of glucose transport induced by altering carbohydrate content of the diet is markedly different from the rapid effect caused by induced hyperglycemia. Three days of high carbohydrate diet feeding were required before an increase in glucose transport was seen suggesting that new carriers be synthesized and not merely recruited from existing pools (Cheeseman and Harley, 1991).

Regulation of the expression of the basolateral transporter GLUT2 has not ben examined extensively in models of intestinal adaptation. Studies have demonstrated, however, that GLUT2 mRNA levels are increased in rats after induction of diabetes with streptozotocin (Miyamoto et al, 1991; Burant et al, 1994).

As mentioned previously, glucose may also pass from the intestinal lumen to serosa via the paracellular pathway. Tight junctions, at one time believed to be static are now known to be dynamic structures subject to regulation. Tight junction resistance can be reduced substantially with the application of glucose to the mucosal surface on the intestine (Asitook et al, 1990). Studies suggest that the paracellular pathway accounts for 30% of absorbed glucose when luminal glucose concentrations are relatively high (Asitook et al, 1990; Pappenheimer & Reiss, 1987). Others, however, question the importance of this pathway under normal physiological conditions (Ferraris et al, 1990). The quantitative relevance of this route of glucose absorption therefore remains in question.

4.1.3 SIGNALS FOR REGULATION

The signals involved in initiating and sustaining enhanced glucose uptake are unknown. Glucose appears to be the most direct signal but hormones and other trophic factors appear to be involved. Clearly the nonspecific regulation of glucose transport seen in pregnancy, lactation and intestinal resection involves hormones and other trophic factors (Hanson et al, 1977; Williamson and Malt, 1981). Gastrin, glucagon, insulin,

cholecystokinin, secretin, prolactin, enteroglucagon and EGF are all possible candidates in enhancing glucose uptake (Johnson, 1976; Rudo and Rosenberg, 1973; Caspary, 1973, Hughes et al, 1981; Weser et al, 1981; Campbell and Fell, 1964; Jacobs et al, 1981; Opleta-Madsen et al, 1991).

The role of humoral signals also extends to the specific mechanisms of upregulated glucose absorption. Intestinal glucose transport is modulated by plasma insulin levels (Westergaard, 1989). Both low and elevated plasma insulin, but not glucose levels, increase maximal rates of intestinal glucose transport. Hypoinsulinemia exerted a larger effect than hyperinsulinemia. In the streptozotocin induced diabetic rat, glucose transport rates and phlorizin binding to BBM vesicles were significantly increased (Fujii et al, 1991). Insulin treatment normalized BBM glucose transport rate and suggests that in addition to the increased levels of glucose in the blood or intestine, insulin partially regulates the increases in transporter expression.

Only recently has the role of other hormones in regulation of intestinal glucose transport been elucidated. Glucose infused into the ileum rapidly increases glucose absorption in the jejunum with no change in ileal glucose transport (Debnam, 1985). Conditions such as resection, gastrectomy and dumping syndrome result in larger than normal amounts of glucose reaching the ileum. The distinct increase in plasma levels of enteroglucagon in these states (Besterman, et al 1982; Macro et al, 1972; Bloom et al, 1972) point to an important role for these hormones in adaptation of glucose transport. The increased release of enteroglucagon may act to enhance glucose absorption in the proximal small intestine.

Enteroglucagon is a post-translational product resulting from the proglucagon precursor. GLP-2 is another proglucagon-derived peptide. Cheeseman & Tsang (1996) recently reported an increase in jejunal glucose uptake following perfusion of the intestine with GLP-2. The role these hormones and others play in the changes observed in intestinal

adaptation in diabetes remains to be elucidated.

4.2 Autoimmune Diabetes and Intestinal Adaptation

Investigations into the etiology of IDDM suggest that both genetic and environmental factors are involved. The fact that identical twins show a concordance rate of less than 50% for IDDM (Barnett et al, 1981) underscores the importance of targeting environmental factors as agents amenable to preventative measures. Discovery of the spontaneously diabetic BB (Biobreeding) rat, possessing many of the features of human type I diabetes has provided a unique model to test the role of dietary components in the induction of the disease (Like et al, 1982; Marliss et al, 1982). Composition of diet as well as timing and duration of exposure to diet appear central to the relationship between environmental factors and diabetes.

4.2.1 Diet composition

Animal Studies:

When fed complex Purina^{R} chow containing a variable mixture of proteins derived primarily from grains, BB rats exhibited a 50% diabetes incidence (Scott et al, 1988). When maintained on a semi-purified diet (SP) (AIN-76) in which the protein source was either 20% casein or 20% soy protein, diabetes incidence was reduced (Hoorfar et al, 1991).

Of great interest in this study is the frequency of insulitis. BB rats fed a plant protein mixture had the highest insulitis frequency (Hoorfar et al, 1991). Although a direct relationship has never been demonstrated, insulitis is generally thought to be a prerequisite for diabetes development. What factors initiate this infiltration of the islets with immune cells is not known but evidence exists that the autoaggressive attack by T cells is mediated by an antigen originating from the pancreas (Ihm et al, 1991).

Transplantation of normal islets from Wistar-Furth rats still results in an immune attack in BB rats, pointing to the presence of an antigen that is normally expressed in pancreatic islets (Rossini et al, 1985). Endogenous secretion of insulin may potentially be a link to the onset of diabetes. By administering exogenous insulin to BBdp rats, the incidence of diabetes can be reduced (Mordes et al, 1987). Conversely, increasing insulin secretion by administration of cyclophosphamide accelerates the progression of β-cell destruction (Mordes et al, 1987; Kurasawa et al, 1993).

HUMAN STUDIES:

Many of the studies addressing the role of milk proteins in the induction of diabetes are retrospective studies on breast feeding practices (Kostraba et al, 1993; Mayer, 1988). Selecting cases from the Colorado IDDM Registry, Mayer (1988) found that subjects with IDDM were 30% less likely to have ever been breast fed as infants than healthy controls. Decreased exposure to breast milk suggests increased exposure to alternate infant formula and solid foods which are potentially diabetogenic. One of the first studies to incorporate both genetic and environmental data in studying IDDM etiology was performed by Kostraba et al (1993). Individuals were defined as high and low risk by an HLA molecular marker. Early exposure to cow's milk was not associated with elevated risk for IDDM in low risk individuals. Individuals with a high risk marker however were strongly associated with early exposure to cow's milk. Similar findings were found for early exposure to solid foods.

Karjalainen et al (1992) recently proposed that sensitization and development of immune memory to cow's milk protein is the initiating step in IDDM etiology. Sensitization would have to occur very early with exposure to cow's milk before gut cellular tight junction closure or during an infection which might compromise intestinal barrier function. After sensitization the potential for immune mimicry resulting in B-cell destruction exists. This hypothesis comes from the observed amino acid similarity between a core sequence of bovine serum albumin and a HLA major histocompatibility

complex II region encoding for HLA-DQB1 and a β-cell surface protein. Milk proteins, however, do not appear to be the sole diabetogens because their removal from diets in rat studies do not avert diabetes onset (Daneman et al, 1987).

4.2.2 INTERPRETING PLANT STUDIES

Interpretation of plant protein studies is difficult due to the impure nature of the plant protein sources added to SP diets. Up to 75% of their composition was as complex carbohydrate (Hoorfar et al, 1991; Hoorfar et al, 1993). To date the significance of plant proteins versus complex carbohydrate in the pathogenesis of IDDM in the BB rat is unknown.

Significantly higher levels of dietary fiber and starch are found in chow diets compared to SP diets (Scott et al, 1985). The fact that SP diets do not completely suppress the induction of diabetes points to the importance of addressing the effect of CHO type on the BB rat. The presence of starch which slows glucose absorption (Wolever, 1991) and can reach the large intestine in significant amounts (Stephen, 1991) may have an association with the incidence of diabetes. Again the observation that addition of 12% crude protein wheat flour (76% complex CHO) was diabetogenic (Hoorfar et al, 1993), but addition of 10% gluten to SP diets was not, provide indirect evidence that differences in carbohydrate type may be implicated in disease pathogenesis. McBurney and Thompson (1989; 1990; 1991) were able to demonstrate that dietary CHO intake affects the amount of fermentable CHO reaching the large intestine and that different rates and extent of fermentation exist for complex CHO. End products of large bowel fermentation, SCFA, increase intestinal cell proliferation and gastrointestinal digestive development (Sakata, 1989; Koruda et al, 1988; Henning, 1985). Owing to the fact that wheat products, containing fermentable CHO are encountered at weaning, the role of these substances in diabetogenesis should be further investigated.

4.2.3 *Timing and Duration of Exposure In Experimental Diabetes*

Whether the protective effect of a semi-purified (SP) diet is due to the presence of a specific protective component or conversely the absence of a diabetogenic component is yet unknown. It is known, however, that the timing of initial exposure and duration of the exposure influence the protective effect of SP diets. The time frame associated with weaning (20-30 d) appears to be a critical `window' in which exposure to protective diet must occur (Issa-Chergui et al, 1988; Scott and Marliss, 1991). If initial exposure is delayed until 30 d, incidence of diabetes in rats does not differ from chow-fed animals (Issa-Chegui et al 1988). Delayed exposure to chow diets (from 21-50 d of age) merely shifted onset to a later age with incidence ultimately the same as the group fed chow diets from weaning (Scott and Marliss, 1991). Further support for the importance of duration is the delayed progression and decreased incidence of disease in those animals fed the SP diet until 100 d and then switched to chow (Scott and Marliss, 1991).

In the life span of an individual, major demands are placed on the gut to adapt to prevailing conditions. Although the intestine is subject to intense antigenic challenges of dietary origin throughout life, of particular interest is the period surrounding weaning. The developing gut is forced to adapt to a diet radically different in composition from that of maternal milk. The suckling period exposes the intestine to a relatively constant liquid diet high in protein and fat and low in carbohydrate. At and beyond weaning the intestine is exposed to a far more variable solid diet generally low in fat and protein and high in carbohydrate including fructose, fiber and starch (Toloza and Diamond, 1992). Concurrent dramatic ontogenic changes are seen in the morphology and enzymology of the small intestine of the infant rat in the third week of postnatal life (Henning, 1981).

Relatively little is known regarding the factors affecting the development of the intestine in the autoimmune BB rat, particularly surrounding the time of weaning. The fact that the drug, cyclosporin A, often used to delay the onset of autoimmune diabetes in rats causes a

delay in the maturation of the small intestine during weaning in the rat (Cummins et al, 1989) suggests that changes occurring in the intestine play a crucial role in disease pathogenesis. As well, it has been shown that rats weaned to chow diets demonstrate higher rates of growth than those weaned to semi-purified diets (Elliot & Martin, 1984; Hoorfar et al, 1992). Pedersen et al (1994) showed that the risk of developing diabetes was greatest in the animals which displayed the highest body weight at 10-40 days of age. Changes in the absorption of nutrients from the intestine of diabetic prone animals and the subsequent effect on pancreatic function may play a role in the ultimate development of the disease.

5 Dietary Fiber and Glycemic control

The term dietary fiber generally refers to the non-starch polysaccharides and lignin present in plant products. Dietary fibers normally escape digestion and are fermented in the colon by bacteria to produce acetate, propionate and butyrate. SCFA derived from fermentation of colonic carbohydrate appear to be well absorbed by the healthy colon and play an important role in colonic epithelial cell metabolism (Cummings, 1981; Bond et al, 1980; Fleming & Arce, 1986).

5.1 Physical form and small intestine effects

The idea that fiber might have beneficial effects on glycemic control in diabetes first emerged around 1976 (Jenkins et al, 1976; Kiehm et al, 1976). Metabolic response to a glucose challenge in humans has shown that high fiber supplemented diets flatten the glucose curve and can actually decrease fasting insulin and glucose levels (Miranda & Horwitz, 1978; Pastors et al, 1991; Fukagawa et al, 1990). High fiber diets have also been reported to improve insulin sensitivity of the body's peripheral tissues and thereby reduce insulin dosage (Anderson, 1986; Fukagawa, 1990). The precise mechanisms responsible for the benefits conferred to diabetic subjects remain unclear.

Guar gum forms a viscous gel and appears to impair glucose absorption (Jenkins et al, 1978; Holt et al, 1979). Many explanations of how a viscous gel can diminish glucose absorption after a glucose load exist (Nuttal, 1993). They include: 1) an effect on gastric emptying; 2) an effect on the diffusion of glucose toward the brush border of the intestine; 3) a change in the unstirred water layer adjacent to the mucosa in the intestinal lumen; 4) an effect of the convective transfer of both glucose and water toward the brush border; 5) an effect on the rate of enzymatic digestion of foods in the intestinal lumen; and 6) an effect on the release of regulatory gut hormones that regulate motility and secretory activity of the intestine. Unfortunately, to date none of the mechanisms alone or in combination fully explain the glucose normalizing effects of fiber.

5.2 ROLE OF GASTROINTESTINAL HORMONES

Speculation that the mechanism responsible for improved glucose tolerance may extend beyond that conferred by the physical form of dietary fiber stem from reports of a "second meal effect" of fiber. The ability of dietary fiber ingested in one meal to affect glucose rise after a second fiber-free meal is called the second meal effect. Reports of second meal effects of fiber ingestion have been shown with guar (Jenkins et al, 1980; Trinick et al, 1986) and pectin (Pastors et al, 1991) supplementation. Not only are post-prandial glucose concentrations and serum insulin levels lowered after a meal with psyllium but this effect extended to a meal 5 h later as well (Pastors et al, 1991). Second meal effects after lunch showed an impressive 31% reduction in post-prandial glucose elevation relative to placebo. The mechanism of action remains unknown.

In a study assessing the long term effects of guar gum on subjects with NIDDM, Groop et al (1993) report a peculiar rise in C-peptide excretion. Differences between insulin and C-peptide concentrations were seen already in the fasting state and remained unchanged in the postprandial period. Measurements of C-peptide are generally thought to be a better estimate of insulin secretory rate than peripheral insulin measurements. Elevated

C-peptide concentrations would seem to represent a true enhancement of insulin secretion. The possibility exists that improved insulin secretion may be partly responsible for the improved glycemic control during guar gum therapy. If modest improvement in glucose control were to act over a prolonged period of time, this improvement might release the inhibitory effect of hyperglycemia on insulin secretion (Yki-Jarvinen, 1990; Rossetti et al, 1987).

Groop et al (1993) suggest that increased hepatic extraction of insulin might explain the unchanged insulin concentrations together with a decreased ratio of insulin to C-peptide throughout the postprandial period. Although insulin and C-peptide are secreted from the β-cells in equimolar amounts (Rubenstein et al, 1969) different mechanisms are responsible for their clearance from plasma. As much as 50-60% of insulin is extracted by the liver during first passage (Jaspan & Polonsky, 1982). C-peptide on the other hand is degraded in the kidneys (Hendriksen et al, 1987). The decreased ratio of insulin to C-peptide seen in the study by Groop et al (1993) is explained by an increase in hepatic insulin extraction. Only recently has it been suggested that the effects on glycemia and insulin secretion are mediated via gastrointestinal factors. Two of the most likely candidates are gastric inhibitory polypeptide (GIP) and GLP-1.

SUMMARY

The effect of dietary fiber on proglucagon mRNA and post-prandial secretion of GLP-1 is unknown. The concept of using fermentable fiber to stimulate proglucagon gene expression would be a novel means of regulating GLP-1 production and release in diabetic subjects. Considering that the main sites of GLP-1 production are the ileum and colon, it follows that gut fuels reaching these segments of the intestinal tract should be considered as potential regulators. In view of the beneficial effects of fiber in diabetic subjects we hypothesize that the fermentation products of fiber, SCFA, may be a signal regulating GLP-1 production. The timely discovery of the mechanistic link between diet

and GLP-1 regulation remains as of yet hidden.

6 *AIMS AND SCOPE OF RESEARCH*

It is well accepted that dietary fiber is able to improve glucose homeostasis in diabetic subjects. Therapeutic advantages of increased dietary fiber include lower exogenous insulin requirements, lower fasting and postprandial plasma glucose and improved glycemic control (Vinik & Jenkins, 1988). Until recently it was thought that the more soluble fibers slowed glucose absorption by increasing the viscosity of the luminal contents. Studies, however, demonstrating improvements in oral glucose tolerance after an overnight fast when patients had consumed fiber long term led to the hypothesis that fiber does not need to be physically present in the intestine to elicit its benefits and that intestinal adaptation may be involved. Studies examining the effect of fiber on the molecular and functional changes occurring in the insulinotropic gut hormone, proglucagon, are warranted.

Whereas the addition of fiber to the diet of diabetic subjects in adulthood appears beneficial, the feeding of diets that contain fiber at weaning is associated with higher incidence of diabetes in the autoimmune BB diabetes prone rat. The association with increased insulitis in those rats fed cereal based diets suggests that the potent insulin secretagogue, GLP-1 may play a role in disease pathogenesis. Increased growth in the early weaning period is also associated with increased disease incidence (Pedersen et al, 1994) and the effects that fiber may have on intestinal growth and nutrient absorption in the BBdp and BBn rat are unknown. Examination of the gene expression of gastrointestinal hormones and glucose transporters will attempt to achieve a mechanistic understanding of the intestine's role in disease pathogenesis.

The potential use of GLP-1 as a therapeutic agent in NIDDM warrants the examination of the actions of GLP-1 in key glucose utilizing tissues. The recent demonstration of

binding of GLP-1 to skeletal muscle suggests that a hormone produced and secreted from the gastrointestinal tract may influence disposition of absorbed nutrients in peripheral tissues. Basic physiological data regarding the actions of GLP-1 is limited. In addition to the understanding of intrinsic basic science this research will ultimately provide practical information on the optimum fiber formulation in diets attempting to improve glucose homeostasis.

The objectives of this research are:

1) to determine the role dietary fiber and proglucagon-like peptides play in glucose homeostasis in normal and diabetic adult animals;
2) to characterize the effect of diet on proglucagon and glucose transporter gene expression in the gastrointestinal tract of 7-30 day old diabetes prone and normal BB rats;
3) to characterize the actions of the intestinal hormone, GLP-1, on skeletal muscle glucose uptake.

These objectives will be achieved by testing the following hypotheses:

Hypothesis 1: Consumption of diets high in fermentable fiber upregulates proglucagon gene expression and secretion of GLP-1 and insulin in normal and diabetic adult rats.

Chapter 2 investigates the effect of a high fiber diet on proglucagon mRNA and secretion of GLP-1 and insulin. Chapter 3 describes the role that a more physiological level of dietary fiber plays in altering proglucagon expression and intestinal glucose uptake. Chapter 4 examines the effect of a more fermentable fiber on the balance between glucose

absorption and glucose disposal in diabetic animals.

Hypothesis 2: Consumption of diets high in fermentable fiber between 21 and 30 days of age increases proglucagon and glucose transporter mRNA abundance in BBn and BBdp animals.

Chapter 5 describes changes in the ontogeny of proglucagon and glucose transporter mRNA and intestinal growth in chow fed BBn and BBdp animals. The BB rat is a model of autoimmune associated diabetes. Chapter 6 examines the effect of weaning diets on proglucagon and glucose transporter mRNA in BBdp and BBn animals.

Hypothesis 3: GLP-1 stimulates insulin-stimulated glucose uptake by skeletal muscle *in vitro*.

Chapter 7 characterizes the actions of GLP-1 on muscle glucose uptake in an *in vitro* model.

Chapter 8 summarizes the findings of these hypotheses and provides insight into the lessons learned through accomplishment of the specific objectives.

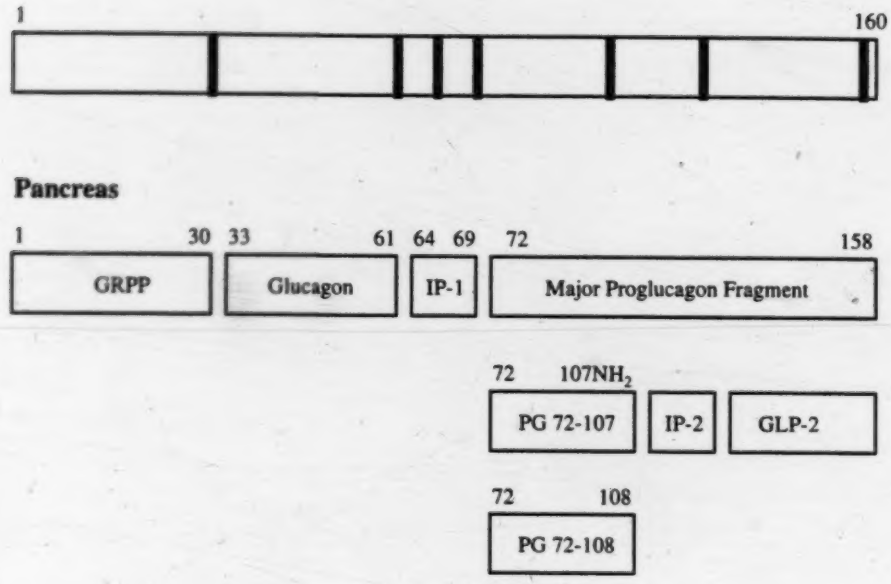

Figure 1.1 Post-translational processing of proglucagon in the pancreas and intestinal L-cells. Enzymatic cleavage occurs at positions indicated by numbers. Adapted from Fehmann & Habener, 1992.

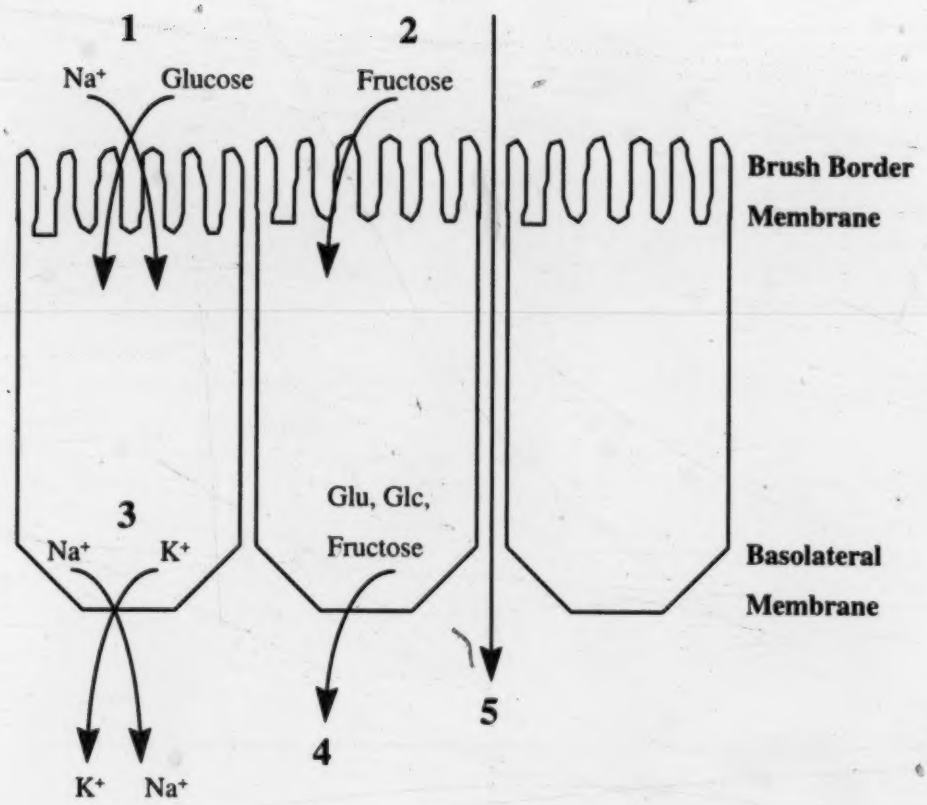

Figure 1.2 Mechanisms of intestinal hexose transport. 1) Na^+-dependent glucose transporter, SGLT-1; 2) Na^+ -independent glucose transporter, GLUT5; 3) Na^+-K^+ ATPase; 4) Na^+ -independent glucose transporter, GLUT2; 5) Paracellular route of absorption. Adapted from Philpott et al, 1992.

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CHAPTER 2

DIETARY FIBER MODULATES INTESTINAL PROGLUCAGON mRNA AND POSTPRANDIAL SECRETION OF GLP-1 AND INSULIN IN RATS[1,2]

INTRODUCTION

It is estimated that > 50% of post-prandial insulin secretion is triggered by intestinal peptide hormones (McIntyre et al, 1964; Hampton et al, 1986). This response is attributed to the incretin effect and is defined as the action of peptide hormones released by the gut in response to oral nutrients to stimulate insulin release in physiologically relevant concentrations. Convincing evidence exists that the truncated form of GLP-1, GLP-1-(7-37) is a physiologically important incretin (Mojsov et al, 1987; Holst et al, 1987) and is produced by the human colon (Deacon et al, 1995). In the presence of elevated blood glucose, GLP-1 stimulates the release of insulin by interacting with specific receptors on pancreatic β-cells. In addition to potentiating glucose induced insulin secretion, GLP-1 also stimulates proinsulin gene expression and proinsulin biosynthesis (Drucker et al, 1994). By stimulating insulin release and increasing insulin-independent glucose disposal, GLP-1 enhances glucose tolerance (D'Alessio et al, 1994). The potent actions of this hormone on carbohydrate metabolism make it potentially applicable in the treatment of non-insulin dependent diabetes mellitus.

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[1] A version of this chapter has been published. Raylene A. Reimer & Michael I. McBurney. Endocrinology 1996; 137:3948-3956

[2] This work was presented in part at Experimental Biology 95, April 1995, Atlanta, Georgia and published in part in abstract form [Reimer RA, McBurney MI, Dietary fiber modulates proglucagon-derived gene expression and insulin secretion in rats. FASEB Journal 9(4):A728]. This work was also presented in part at Canadian Federation of Biological Societies, June 1994, Montreal, Quebec and published in abstract form [Reimer RA, McBurney MI, Dietary fiber modulates intestinal glucose gene expression in rats. CFBS 37:A177].

Proglucagon, the mammalian glucagon precursor, is a 160 amino acid polypeptide encoded by the glucagon gene (Bell et al, 1983). The precursor is produced both in the alpha cells of the islets of Langerhans and in the L cells of the intestinal mucosa (Mojsov et al, 1986; Orskov et al, 1986). Intestinal L cells produce predominantly glicentin, which corresponds to amino acids 1-69 of the proglucagon precursor (PG 1-69), a C-terminal extended form of glucagon (oxyntomodulin; PG 33-69), GLP-1 (PG 78-108), GLP-2 (PG 126-158) and intervening peptide 2 (PG 111-123). Post-translational cleavage results in the secretion of amidated and glycine extended GLP-1 which have similar potency in terms of insulin secretion. In rats, one third of the GLP-1 immunoreactivity corresponds to glycine-extended GLP-1 and two thirds is in the form of amidated GLP-1 (Mojsov et al, 1990). In humans this partitioning is approximately 20% and 80% respectively (Orskov et al, 1994). Although a gradient of L-cells is present throughout the gastrointestinal tract, the principal production of GLP-1 is concentrated in the L-cells of the distal small intestine and colon (Holst, 1994; Deacon et al, 1995)). Regulation of the production/secretion of GLP-1 may be subject to changes in intestinal mass and function. These morphological and functional changes, or adaptation, can occur with age, resection, dietary manipulation and in certain disease states such as diabetes mellitus (Goodlad et al, 1989; Taylor et al, 1990; Pillion et al, 1988). Indeed, D'Alessio et al (1996) suggested that differences in basal and stimulated GLP-1 secretion among individuals may account for some of the variation in levels of glucose before, as well as after, eating. These differences could be amplified by adaptations associated with diet.

Dietary management of Diabetes Mellitus includes recommendations for increasing levels of complex carbohydrate and dietary fiber (American Diabetes Association, 1987). Therapeutic advantages of increased dietary fiber include lower exogenous insulin requirements, lower fasting and postprandial plasma glucose and improved glycemic control (Vinik & Jenkins, 1988). In studies involving long-term ingestion of fiber, improvements in glycemic control are seen with an oral glucose tolerance test after an overnight fast (Groop et al, 1993). Decreased or unchanged insulin responses to test

meals have been reported (Pastors et al, 1991) but with long term ingestion of fiber, serum C-peptide response increased whereas serum insulin response remained unchanged. Increased C-peptide suggests a stimulation of insulin. The precise mechanisms involved in improved glucose tolerance are not known but the effect may be mediated via gastrointestinal hormones.

Dietary alterations, such as the addition of certain readily fermentable fibers to an elemental diet cause a significant proliferative effect in the colon and distal small intestine (Jacobs & Lupton, 1984). The effect does not simply reflect the benefits of bulk in the lower gastrointestinal tract but appears to also be related to the production of short chain fatty acids (SCFA) from microbial fermentation of fiber (Kripke et al, 1989; Sakata, 1987; Rombeau & Kripke, 1990; Goodlad et al, 1987). Crypt cell production rates are significantly increased when fermentable fibers are ingested, suggesting a role for SCFA in the proliferative response. Additionally, the ingestion of fermentable dietary fiber seems to enhance the secretion of the gut trophic hormone, enteroglucagon (Southon et al, 1987; Gee et al, 1996).

The three major SCFA, acetate, propionate and butyrate, once absorbed are metabolized by the cecal and colonic mucosal epithelial cells. Butyrate is recognized as an important respiratory fuel for the colon in preference to acetate and propionate as well as glutamine, glucose and ketone bodies, fuels commonly utilized by intestinal epithelial cells (McIntyre et al, 1993; Marsman & McBurney, 1995). Although incubation of a primary fetal rat intestinal culture with sodium butyrate for up to 2 hours did not stimulate secretion of glucagon-like immunoreactive peptides (Brubaker, 1988), addition of sodium butyrate to a pancreatic cell line markedly increased glucagon mRNA levels after 6 hours (Philippe et al, 1987). Glucagon and insulin gene transcription increased by 9.3- and 5.3-fold respectively. Alterations in secretion and gene expression may be modulated via different mechanisms in the L cell and may explain the differences seen between these two *in vitro* studies. The ability of fermentable carbohydrates to increase plasma

enteroglucagon *in vivo* has been documented (Goodlad et al, 1987; Gee et al, 1996). It remains to be elucidated if dietary fiber and possibly SCFA similarly regulate gene expression of gastrointestinal hormones.

The hypothesis that dietary fiber regulates gene expression and secretion of the gastrointestinal peptide hormone, GLP-1, was tested in the present study by comparing effects of an elemental and 30% (wt/wt) fiber diet on proglucagon mRNA. Measurements of plasma GLP-1-(7-37), insulin, C-peptide, glucagon and glucose were determined at 30 minutes post-oral glucose. Differences in concentration and total amount of SCFA in cecal and colonic contents were assessed as well.

MATERIAL AND METHODS

Animals and Diets Female Sprague-Dawley rats (190-220 g) were obtained from the University of Alberta Health Sciences Laboratory Animal Services colony and housed in a temperature and humidity controlled room with a 12 hour light/dark cycle. Animals were housed in individual wire mesh-bottomed cages. The protocol was approved by the University of Alberta Animal Welfare Committee.

Animals were maintained on a nonpurified diet (Rodent Laboratory Diet PMI #5001®, PMI Feeds, Inc. St. Louis, MO) prior to the experimental period. During the experiment animals consumed either a fiber free elemental diet (ICN Chemically Defined Diet #960346®, ICN Biomedicals, Mississauga, Canada) or an elemental diet supplemented with 30% fiber (wt/wt, 5% alphacel + 25% Fibrad®, ICN, Mississauga, Canada; Ross Laboratories, Columbus, OH) for 14 d. Animals had free access to the diet during the experimental period. Composition of the experimental diets is given in **Table 2.1**. When diets are diluted by addition of fiber, rats increase their food intake to achieve similar energy intakes (Lupton & Marchant, 1989; Dowling et al, 1967). Thus the 30% fiber diet was formulated so that animals eating to a constant digestible energy intake would have identical nutrient and nutrient:digestible energy intakes on both diets.

Oral Glucose Gavage and Sample Collection After an overnight fast all animals were given 50% (wt/v) dextrose by gavage at a dose of 2 g glucose/kg. At 30 min post gavage animals were anesthetized and blood taken by cardiac puncture. The small intestine was partitioned under tension (15g) into three equal segments (duodenum, jejunum and ileum). A 3 cm segment of distal ileum and proximal colon was immediately excised, flushed with ice cold saline, immersed in liquid nitrogen and stored at -72°C for later mRNA analysis. Cecal and colonic contents were extruded into vials for later SCFA analysis. One hundred microliters of 0.17% (wt/v) phosphoric acid and 1 ml of water were added to the contents. Empty ceca and colons were rinsed and weighed.

Isolation of Total RNA Total RNA was isolated from each segment using a procedure described by Chirgwin et al (1979) with modifications. Briefly, approximately 0.3 g of intestine was added to guanidium isothiocyanate (GIT) containing 0.5% (wt/v) Antifoam A and homogenized at top speed for 20 sec. To each sample 0.2 ml NaOAc (2M), 2 ml buffered phenol and 0.4 ml chloroform/iso-amyl alcohol were added. Tubes were placed on ice for 15 min and then centrifuged at 9000 x g for 20 min at 4°C. DNA will be at the interface with proteins and lipids in the organic (lower) phase. About 2.2 ml of the RNA containing upper phase was recovered to a new microcentrifuge tube. Equal volume ice cold isopropanol was added to precipitate the RNA overnight at -20°C. Microfuge tubes were centrifuged at 9000 x g for 20 min at 4°C, the liquid drawn off and the pellet resuspended in 0.6 ml GIT. Tubes were placed in a 60°C water bath to dissolve the pellet. Ice cold isopropanol (0.6 ml) was again added and RNA allowed to precipitate for 1 hour at -20°C. Microfuge tubes were centrifuged 20 min at 9000 x g and the liquid drawn off. A standard phenol/chloroform/iso-amyl alcohol (25:24:1) extraction was performed by redissolving pellet in 400 ul TE (10mM Tris-Cl, 1mM EDTA). RNA was ethanol precipitated overnight and pellet dissolved in 400 ul/g tissue of STE (10mM Tris-HCl, 5mM EDTA, 0.1% (wt/v) SDS). Quantity and purity of RNA was determined by ultraviolet spectrophotometry at 260, 280 and 230 nm.

Ribonuclease Protection Assay Glucagon mRNA in all samples was measured in an RNase protection assay procedure as described by Gilman (1987) with modifications. The 440 bp glucagon cRNA probe (Taylor et al, 1990) was a gift from Peter J. Fuller of Prince Henry's Institute of Medical Research in Melbourne, Australia. Aliquots of 15 µg sample total RNA were hybridized overnight with 3 x 10^6 dpm of the [^{32}P]-CTP (Du Pont Canada Inc, Markham, ONT) labelled rat glucagon riboprobe and then incubated for 1 h at 30°C in 350 ul of digestion buffer containing 10 mmol/l Tris-HCl, 300 mmol/l NaCl, 5 mmol/l EDTA, 0.04 g/l RNase A (Pharmacia LKB Biotechnology, Uppsala, Sweden) and 0.002 g/l RNase T1 (Sigma Chemical Co., St. Louis, MO, U.S.A). The protected fragments were then size fractionated by electrophoresis on a denaturing 6% polyacrylamide/7M urea sequencing gel using 40 W constant power and approximately 1000 V (Little & Jackson, 1987). Following electrophoresis, the gel plates were separated and the gel rinsed in 10% glacial acetic acid (v/v) and 10% methanol (v/v) for 15 min. After draining for 15 min, the gel was transferred to 3MM Whatman filter paper (Whatman International, Maidstone, Kent, U.K.), heat sealed into a plastic bag and exposed to Kodak XAR 5 film at -70°C using an intensifying screen (Dupont Canada, Mississaugua, Ontario, Canada).

Controls

To confirm the authenticity of protected fragments in the protection assay, samples containing only yeast tRNA, in amounts equal to that used in all other RNA samples, were included in each assay and treated exactly as all other RNA samples. There is no mRNA present in yeast tRNA and, consequently, any protected bands detected reflect non-specific binding of the probe. The Riboprobe alone (6000 dpm) was run in a separate lane and served as a positive control. A dose response curve of 10, 15, 25 and 35 µg total RNA from one animal was treated as all other samples and run on each gel as well. This ensures the probe was present in molar excess over the target fragment in the hybridization reaction and the intensity of the protected fragment directly proportional to the amount of complementary RNA in the sample mixture.

Consistent with other published work using RNase protection assays (Dardevet et al, 1994; Winesett et al, 1995), and the nature of the assay, separate probes such as actin were not used as loading controls. Like Winesett et al (1995) we confirmed the integrity of total RNA and loading accuracy by running separate RNA formaldehyde-denaturing agarose gels to visualize 28S rRNA and 18S rRNA bands. Use of 28S rRNA as a control for total RNA loading has been reported to be equivalent or superior to the use of constitutively expressed RNA such as ubiquitin or actin (Jin et al, 1990) and a more reliable and reproducible control since signals of interest can be obscured by the high background often produced with constitutively expressed mRNA such as actin mRNA (Winesett et al, 1995).

SCFA Analysis At the time of analysis, 0.5 ml (3 mg/ml) of isocaproic acid was added as internal standard to each sample. Samples were frozen overnight and then centrifuged in eppendorf tubes until a clear supernatant was obtained. One milliliter of supernatant was run on gas chromatography. (Column: Stabilwax-DA 30m x 0.25mm I.D.; Temp: 120° C to 170°C at 10°C/min; Injector: 170°C; Detector: 190°C; Split ratio: 20:1).

Radioimmunoassays Approximately 8 ml of blood were collected from each rat into a chilled syringe of which 6 ml were collected with the addition of EDTA (1 mg/ml blood) and aprotinin (500 KIU/ml blood, Sigma Chemical, St. Louis, MO). The remaining 2 ml were mixed with 80 ul of Heparin Leo® (1000 i.u./ml, Leo Laboratories Canada Ltd, Ajax, ON) and 120 ul of NaF (0.025 g/ml, Fisher Scientific). Blood was centrifuged at 1,600 x g for 15 min at 0°C and aliquots taken for GLP-1, glucagon and C-peptide determinations from the EDTA samples. The NaF samples were divided to provide aliquots for insulin and glucose determinations. Samples were stored at -70°C.

GLP-1-(7-37)

GLP immunoreactive peptides were extracted from 2.5 ml of plasma using a SEP-COLUMN containing 200 mg of C_{18} (Cat # RIK-SEPCOL 1, Peninsula Laboratories,

Belmont, CA) with Buffer A (0.1% trifluoroacetic acid (Cat # RIK-BA-1, Peninsula Laboratories, Belmont, CA)) and Buffer B (60% acetonitrile (Cat # RIK-BB-1, Peninsula Laboratories, Belmont, CA)) as elution solvents. The extraction was performed according to the protocol provided with the GLP-1-(7-37) RIA kit. The recovery rate of the extracted peptide was 50% using this method. Concentrations of GLP-1-(7-37) were measured using a double-antibody radioimmunoassay kit (Peninsula Laboratories, Belmont, CA, Cat. No. RIK-7123). This kit measures GLP-1-(x-37) with <0.01% crossreactivity with GLP-1-(7-36). The ED_{50} for this assay is 45 pg/tube at a binding of 98.2% (defined as mean total bound minus two standard deviations from mean total bound). The intra-assay coefficient of variance was 6.76%.

C-Peptide

Plasma levels of C-peptide were quantified in a single radioimmunoassay using a commercial rat C-peptide RIA kit (Linco Research, St. Louis, MO). The ED_{50} for this assay is 397 pmol/L at a binding of 98.8% (defined as mean total bound minus 2 standard deviations from mean total bound). The intra-assay coefficient of variance was 2.10%.

Insulin and Glucagon

Plasma insulin and glucagon concentrations were measured at the Muttart Diabetes Research Center, University of Alberta. Insulin was determined using a commercial double antibody radioimmunoassay kit (Linco Research, St. Louis, MO) for rat insulin with a detection limit of <2 μU/ml. Plasma levels of glucagon were determined using a commercial double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Plasma Glucose Determination

Plasma glucose was determined using Sigma Diagnostics Glucose (Trinder) Reagent for the enzymatic determination of glucose at 505 nm (Sigma Chemical, St. Louis, MO).

Statistical Analysis All data are given as mean ± SEM. Differences between treatments were determined using the one-way ANOVA model in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Statistical significance is defined as $p \leq 0.05$.

RESULTS

Diet Intake and Growth

Animals consumed 15.2 ± 0.9 g/d and 19.5 ± 0.9 g/d of the 0% and 30% fiber diets, respectively. These means were significantly different ($p<0.05$). Under in vitro fermentation conditions Fibrad, a moderately fermentable fiber source produced 4.50 mmol SCFA/g organic matter (McBurney, 1991). Digestible energy of the fiber mixture was determined experimentally *in vivo* to be 1.17 kcal/g (Marsman & McBurney, unpublished data). Nutrient intakes, with the exception of dietary fiber did not differ between diets because of the greater intake on the 30% fiber diet. The animals eating the 0% and 30% fiber diets ingested similar amounts of energy (64.9 ± 3.4 kcal/d vs 65.0 ± 3.3 kcal/d), protein (2.7 ± 0.1 g/d vs 2.4 ± 0.1 g/d), lipid (1.5 ± 0.1 g/d vs 1.4 ± 0.1 g/d), sugars (10.1 ± 0.5 g/d vs 9.0 ± 0.5 g/d) and vitamin and mineral mix (0.9 ± 0.04 g/d vs 0.8 ± 0.04 g/d) respectively. No significant differences were found. Animals gained 2.9 ± 0.3 g/d and 3.5 ± 0.3 g/d on the 0% and 30% fiber diets, respectively. Weight gain was not significantly different.

Intestinal Segment Characteristics

Total small intestine weight did not differ between the two groups due to preferential weight gain in the duodenum and jejunum with the 0% fiber diet and in the ileum with the 30% fiber diet (**Table 2.2**). In the distal gut, the 30% fiber diet resulted in a significant increase in mass of the ileum, cecum and colon. Total RNA content of the colon was greater with the high fiber diet compared to the elemental diet (1.9 ± 0.1 mg/g wet tissue vs. 1.6 ± 0.1 mg/g wet tissue) ($p<0.05$).

SCFA Analysis

The concentration of butyric acid was significantly higher (0.03 ± 0.004 mmol/mg wet weight vs. 0.02 ± 0.003 mmol/mg wet weight) in the cecum of the 30% fiber animals ($p<0.05$). As well, animals fed the 30% fiber diet had a greater mass of cecal contents and higher amount (mmol) of the predominant SCFA and summed total SCFA. The effect of fiber on the amount (mmol) of SCFA found in the cecum is shown in **Figure 2.1.** Differences due to diet were found for acetate, butyrate and total SCFA ($p\leq0.05$).

Concentrations (mmol/g wet weight) of SCFA in colonic contents were unaffected by diet. **Figure 2.2** shows the amount (mmol) of predominant and total SCFA in the colon. Only butyrate was increased by the 30% fiber diet ($p\leq0.05$). Although not significant, all other SCFA assayed were consistently higher with the fiber diet.

Proglucagon mRNA

The protected proglucagon mRNA fragment of 340 bases was readily detected in the total RNA from colon and ileal samples. As shown in **Figures 2.3** and **2.4**, statistical analysis of the mean densitometric readings confirm that feeding a 30% fiber diet significantly increases the amount of proglucagon mRNA in the ileum ($p\leq0.05$) but in the colon only reached a 'p' value of 0.07. Ileal levels of proglucagon mRNA in the 30% fiber animals were nearly double those observed in the 0% fiber animals (11.5 ± 0.9 vs. 6.5 ± 0.9 densitometer units). Colonic levels of proglucagon mRNA in the 30% fiber fed animals were approximately 20% higher than those observed in the 0% fiber fed animals (13.4 ± 1.0 vs. 10.9 ± 0.8 densitometer units) .

Radioimmunoassays

Figure 2.5 shows the increased levels of GLP-1 present in the blood of rats fed the 30% fiber diet versus the 0% fiber group ($p=0.02$). Insulin levels were significantly higher in the high fiber fed group (**Figure 2.6**). As observed with the insulin responses, C-peptide levels in rats consuming the 30% fiber diet were significantly higher than the 0% fiber fed

group (**Figure 2.6**).

There was no significant difference in the levels of glucagon between the two groups (**Figure 2.7**). At the 30 min time point no difference was found in plasma glucose levels between the two groups (**Figure 2.7**).

DISCUSSION

Ileal proglucagon gene expression is known to adapt (Bloom & Polak, 1982) with increases occurring after massive small bowel resection (Taylor et al, 1990). As well levels of proglucagon mRNA in the developing ileum increase in the postnatal period, peaking at weaning and then decreasing somewhat before reaching adult levels (Taylor et al, 1990). The present study demonstrates that long term adaptation to dietary fiber modulates proglucagon mRNA abundance and subsequent GLP-1-(7-37) secretion following an oral glucose load.

In this study, animals ate equivalent amounts of nutrients and only dietary fiber intake differed. SCFA contents in the cecum and colon were increased with fiber as expected (Kripke et al, 1989; Sakata, 1987; Rombeau & Kripke, 1990). Indeed, luminal concentrations of SCFA likely underestimate total production because approximately 95% of those produced are absorbed (Cummings, 1995). The predominant SCFA, acetate, propionate and butyrate, account for approximately 90-95% of SCFA production. The localization of L-cells to the distal gut places them in a strategic position to respond to short chain fatty acids. Our results confirm a higher content of SCFA in the cecum and colon of the animals fed the 30% fiber diet which may be responsible for changes in proglucagon gene expression and postprandial GLP-1 secretion. Luminal pectin but not SCFA stimulate release of GLP-1-(7-36)amide in the isolated vascularly perfused colon (Plaisancie et al, 1996). Therefore, we cannot determine if the release of GLP-1 is modulated by SCFA production or the entry of fiber into the large intestine. However,

intravenous infusions of SCFA with total parenteral nutrition (TPN) in rats following massive small bowel resection significantly increased proglucagon mRNA abundance at 3 and 7 days postoperatively (Tappenden & McBurney, 1996).

Increases in gene expression are physiologically meaningful if concomitant alterations are also seen in the resulting peptide. The commercial antibody used in this RIA is directed toward the c-terminal end and thus does not differentiate between pancreatic GLP-1-(1-37) and intestinal GLP-1-(7-37). However, after a meal nearly all of the GLP-1 immunoreactive material is caused by changes in secretion of GLP-1 moities from the small intestine rather than the pancreas (Orskov et al, 1991). Thus, our RIA predominantly reflects the response of the intestinal insulinotropic forms of GLP-1 immunoreactivity rather than the non-insulinogenic pancreatic forms. The acceptance that approximately 70% of GLP-1 secretion from the small intestine of the rat (Mojsov et al, 1990) and 80% in humans (Orskov et al, 1994) is in the amidated form clearly indicates that the newly available RIA for GLP-1-(7-36) amide is the most accurate means of determining GLP-1 in a single RIA. It is probable that the RIA used in this experiment underestimates total small intestinal GLP-1 secretion because GLP-1-(7-36)amide is not quantified. Pancreatic GLP-1-(1-37) is not insulinotropic (Mojsov et al, 1987) so we conclude that the changes observed in plasma insulin and C-peptide concentrations reflect increased intestinal proglucagon expression and GLP-1 secretion.

Plasma concentrations of GLP-1 vary with meal pattern (Orskov et al, 1994). In healthy human volunteers fasting GLP levels are 30-40 pmol/L and rise about twofold after an oral glucose load with a peak occurring at 30 minutes and declining to basal values after 180 minutes (Orskov et al, 1991). In the rat, we observed plasma values of GLP-1-(7-37) in the range of 15-20 pM at 30 minutes after oral glucose. Since the GLP-1 RIA requires 6 ml of whole blood for extraction of the peptide and another 2 ml was required for glucose, insulin, glucagon and C-peptide, we could only obtain one plasma sample per rat. We chose the 30 minute time point when GLP-1 concentrations are elevated (Orskov

et al, 1994) to determine diet-induced differences in GLP-1 secretion. Unfortunately, due to the constraints on blood sampling, it was physically impossible to obtain multiple samples and determine incremental areas under the curve for metabolites of interest. In this study, all animals were gavaged with 50% glucose solutions. D'Alessio et al (1995) have shown that plasma GLP-1 concentrations are increased with luminal nutrients but not water or saline. Our comparisons are only after an oral glucose load. The increase in GLP-1 observed support the findings of Gee et al (1996) where fermentable, but not viscous, carbohydrate result in increased plasma enteroglucagon concentrations.

Studies in the rat insulinoma cell line RIN 1046-38 showed that GLP-1 increased levels of proinsulin mRNA (Drucker et al, 1987). In addition, it has been shown that GLP-1 also stimulates proinsulin biosynthesis in insulinoma cells (Holst, 1994). Consistent with the known biological action of GLP-1, insulin and C-peptide secretion were elevated in animals fed the 30% fiber diet. Many studies have shown that longterm ingestion of fiber improves glucose tolerance. Groop et al (1993), in contrast with some reports (Jenkins et al, 1976) and in agreement with others (Hagander et al, 1984; Aro et al, 1981), did not find changes in serum insulin concentrations, however, in response to oral glucose, C-peptide increased after guar gum treatment. C-peptide measurements provide a better estimate of insulin secretory rate than do peripheral insulin measurements and represent a true enhancement of insulin secretion (Jacobs & Lupton, 1984; Polonsky & Rubenstein, 1984).

In summary, we suggest that reported improvements in glycemic control following adaptation to dietary fiber can be explained by changes in SCFA production which modulates intestinal proglucagon abundance and postprandial GLP-1 secretion. McBurney et al (1995) used the frequently sampled intravenous glucose tolerance test to demonstrate that intravenous infusions of SCFA do not affect insulin sensitivity, glucose disposal or insulin secretion. However, intravenous SCFA do increase proglucagon mRNA abundance following massive small bowel resection (Tappenden & McBurney,

1996). Thus, in demonstrating that SCFA do not directly affect insulin secretion or glucose metabolism, McBurney et al (1995) confirmed previous observations (Hampton et al, 1987) that the enteroinsular axis can be uncoupled by circumventing the intestine with intravenous infusions. In this study, we show that longterm ingestion of dietary fiber by rats ingesting similar amounts of energy, protein, lipid, glucose and vitamins and minerals, stimulates SCFA production, proglucagon mRNA abundance, and increases postprandial GLP-1 , insulin and C-peptide concentrations and propose a mechanism to explain improvements in glycemic control observed with chronic consumption of dietary fiber.

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TABLE 2.1 Composition of the Experimental Diets

Ingredient	0% Fiber	30% Fiber
	g/kg diet	
Amino Acid Mix[1]	178.5	124.95
Corn Oil	100.0	70.0
Glucose	443.0	310.1
Sucrose	221.5	155.05
Alphacel	--	50.0
Fibrad[2]	--	250.0
Mineral Mix[3]	50.0	35.0
Vitamin Mix[3]	7.0	4.9
Digestible Energy[4] **(kJ/g)**	17.5	13.8

[1]Supplied (g/178.5 g mix): L-arginine HCl, 13.5; L-histidine HCl.H_2O, 4.5; L-isoleucine,8.2; L-leucine, 11.1; L-lysine HCl, 18.0; L-methionine, 8.2; L-phenylalanine, 11.6; L-threonine, 8.2; L-tryptophan, 1.7; L-alanine, 3.5; L-aspartic acid, 3.5; L-glutamic acid, 35.0; glycine, 23.3; L-proline, 3.5; L-cystine, 3.5; L-serine, 3.; L-tyrosine, 3.5; L-asparagine, 6.0.

[2]Ross Laboratories, Columbus, OH. Contained a mixture of pea fiber, oat fiber, sugar-beet fiber, xanthan gum and soy lecithin.

[3]Supplied in quantities adequate to meet NRC nutrient requirements.

[4]Digestible energy (DE) was calculated from gross energy of diet components and in vivo DE measurements for the fiber sources.

TABLE 2.2 Effect of Dietary Fiber on Length and Weight of Intestinal Segments

Parameter	0% Fiber	30% Fiber	Significance
Total small bowel length (cm)	143.30 ± 2.29	138.67 ± 2.41	NS
Total small bowel weight (g)	7.87 ± 0.27	7.79 ± 0.27	NS
Duodenum weight (g)	3.11 ± 0.14	2.78 ± 0.14	NS
Jejunum weight (g)	2.70 ± 0.11	2.52 ± 0.11	NS
Ileum weight (g)	2.04 ± 0.12	2.50 ± 0.12	$p<0.05$
Cecum weight (g)	0.63 ± 0.02	0.89 ± 0.06	$p<0.05$
Total colon length (cm)	14.60 ± 0.88	19.00 ± 0.99	$p<0.05$
Total colon weight (g)	0.93 ± 0.09	1.47 ± 0.09	$p<0.05$

Values are mean ± SEM (n=10 rats / treatment). NS = Not Significant. Total small bowel was partitioned under tension with a 15 g weight into 3 equal segments for duodenum, jejunum and ileum.

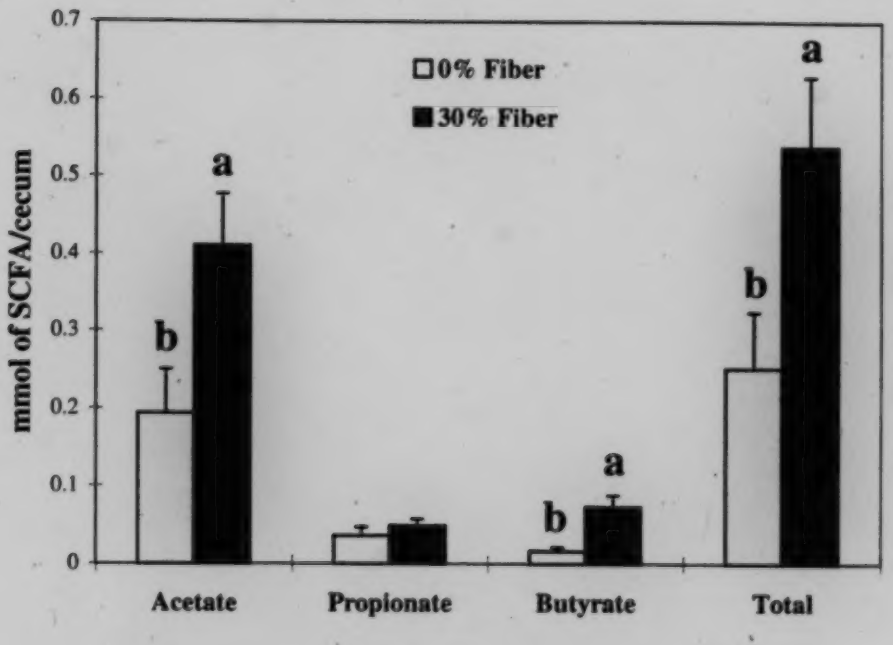

Figure 2.1 Effect of fiber supplementation on SCFA content in the cecum of rats. Values are mean ± SEM (n=6 rats / treatment). Total SCFA are the sum of all individual SCFA analysed (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, caproic). Amount (mmol) of SCFA was obtained by multiplying mass of cecal contents with concentrations (mmol/mg of wet contents). For each individual SCFA, values with different letters are significantly different ($p < 0.05$).

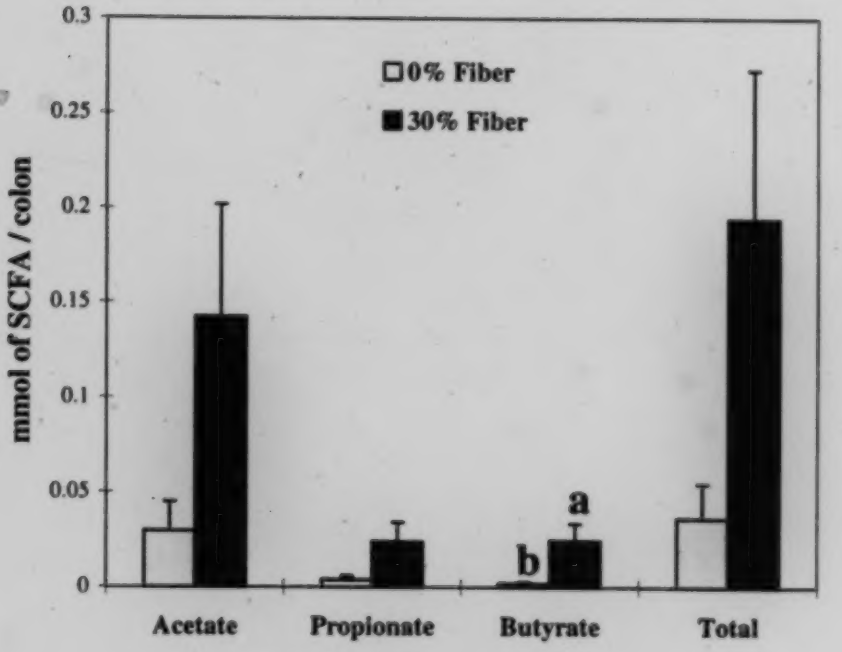

Figure 2.2 Effect of fiber supplementation on SCFA content in the colon of rats. Values are mean ± SEM (n=6 rats / treatment). Total SCFA are the sum of all individual SCFA analysed (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, caproic). Amount (mmol) of SCFA was obtained by multiplying mass of colonic contents with concentrations (mmol/mg of wet contents). For each individual SCFA, values with different letters are significantly different ($p < 0.05$).

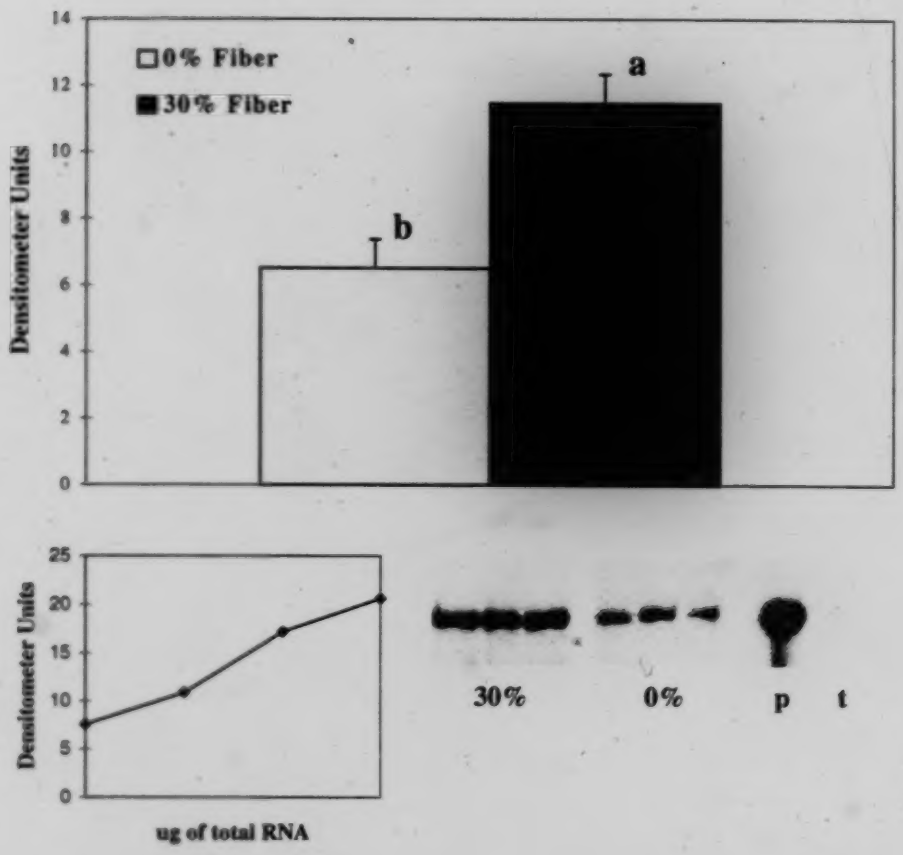

Figure 2.3 Effects of increasing levels of fiber supplementation on ileal proglucagon mRNA expression in rats. Values are mean ± SEM (n=6 rats / treatment). Values with different letters are significantly different ($p<0.05$). Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA and represents an individual animal. See *Materials and Methods* for explanation of controls. 30% = 30% fiber diet; 0% = 0% fiber diet; p = probe alone; t = tRNA.

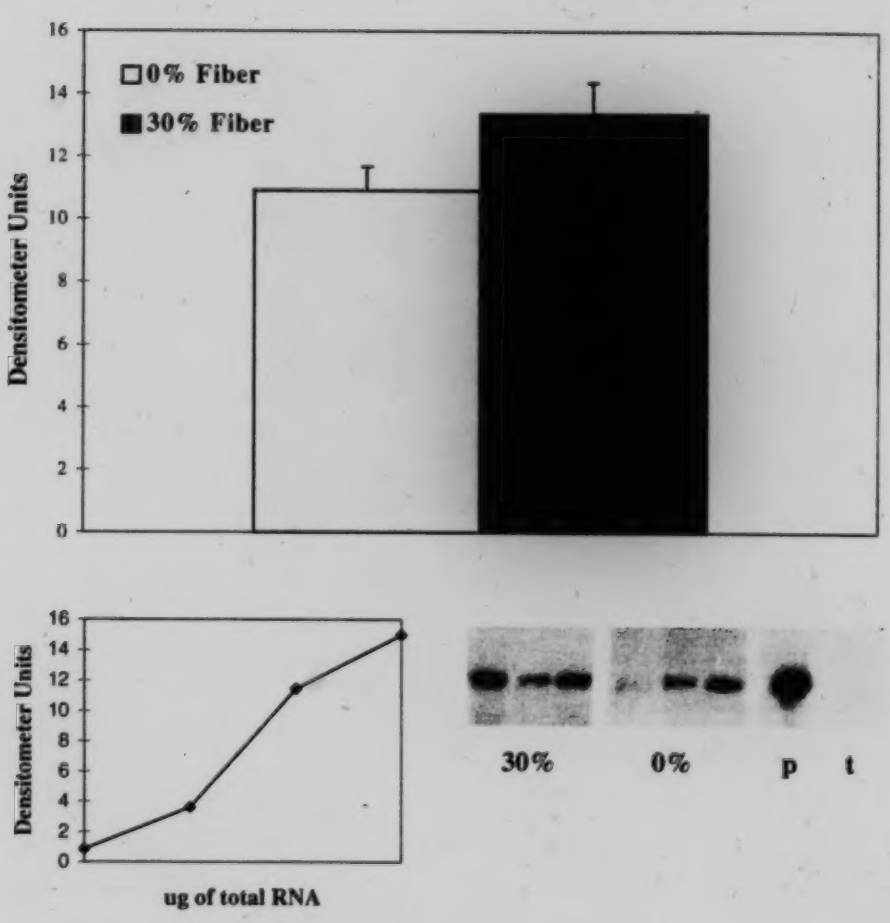

Figure 2.4 Effects of increasing levels of fiber supplementation on colonic proglucagon mRNA expression in rats. Values are mean ± SEM (n=12 rats / 0% treatment; n=8 rats / 30% treatment). No significant differences were found. Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA and represents an individual animal. See *Materials and Methods* for explanation of controls. 30% = 30% fiber diet; 0% = 0% fiber diet; p = probe alone; t = tRNA.

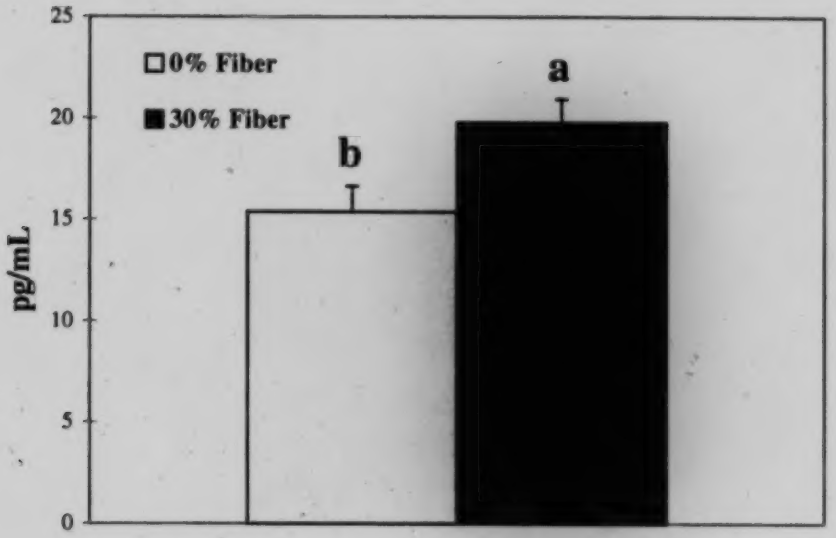

Figure 2.5 Plasma levels of GLP-1(7-37) 30 min post-oral glucose load in rats fed either a 0% or 30% fiber diet. Values are mean ± SEM (n=8 rats / 0% treatment; n=9 rats / 30% treatment). Values with different letters are significantly different ($p<0.05$).

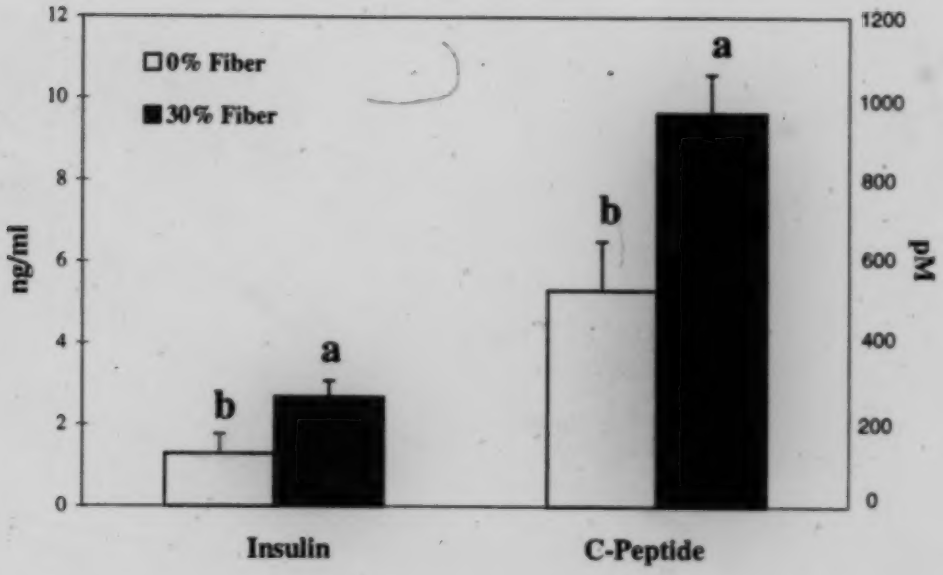

Figure 2.6 Plasma levels of insulin and C-peptide 30 minutes post-oral glucose load in rats fed either a 0% or 30% fiber diet. Values are mean ± SEM (for insulin: n=9 rats / 0% treatment and n=13 rats / 30% treatment; for C-peptide: n=8 rats / 0% treatment and n=13 rats / 30% treatment). For each individual peptide, values with different letters are significantly different ($p<0.05$).

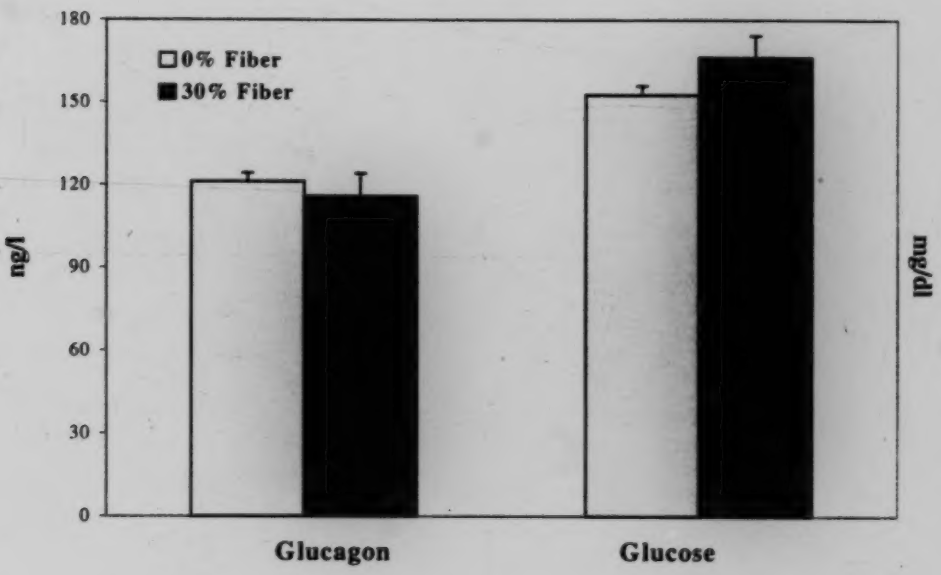

Figure 2.7 Plasma levels of glucagon and glucose 30 minutes post-oral glucose load in rats fed either a 0% or 30% fiber diet. Values are mean ± SEM (for glucagon: n=10 rats / 0% treatment and n=9 rats / 30% treatment; for C-peptide: n=9 rats / treatment). For each individual peptide, values with different letters are significantly different ($p<0.05$).

CHAPTER 3

A PHYSIOLOGICAL LEVEL OF RHUBARB FIBER INCREASES PROGLUCAGON GENE EXPRESSION AND MODULATES INTESTINAL GLUCOSE UPTAKE IN RATS[1,2]

INTRODUCTION

Current recommendations for the dietary management of diabetes mellitus include increasing the levels of complex carbohydrate and dietary fiber in the diet (American Diabetes Association, 1987). Increasing dietary fiber confers such benefits as lower exogenous insulin requirements, lower fasting and postprandial plasma glucose and improved glycemic control (Vinik & Jenkins, 1988). The more highly soluble fibers including pectin, psyllium and guar gum appear to have a greater impact on glucose tolerance due to their ability to slow glucose absorption in the small intestine (Jenkins et al, 1978; Pastors et al, 1991). After long-term ingestion of fiber, however, improvements in glycemia can be recognized even when fiber is not physically present in the intestine i.e. after an overnight fast (Pastors et al, 1991; Groop et al, 1993). Only recently has it been suggested that the effects of dietary fiber on glucose transport, insulin secretion and glycemia may be mediated by changes in gastrointestinal hormones as well.

The addition of fermentable fiber to an elemental diet causes a significant proliferative effect in the colon and distal small intestine (Jacobs & Lupton, 1984). The trophic effect appears to be related to the production of short-chain fatty acids (SCFA) which result from the microbial fermentation of dietary fiber in the gut (Sakata, 1987; Rombeau &

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[1]A version of this chapter has been accepted for publication in *The Journal of Nutrition.*

[2]This work was presented in part at Experimental Biology 97, April 1997, New Orleans, Louisiana and published in abstract form [Reimer RA, McBurney MI, Baracos VE, Tomson ABR, Basu TK, Ooraikul B. Modulation of glucose homeostasis by rhubarb fiber and GLP-1. FASEB Journal 11(3):A3533].

Kripke, 1990). Indeed, both ingestion of a high fiber diet and supplementation of total parenteral nutrition (TPN) with SCFA upregulate proglucagon mRNA (Reimer & McBurney, 1996; Tappenden et al, 1996). It remains to be elucidated if the ingestion of different fiber types also alter proglucagon mRNA abundance.

Proglucagon, synthesized by L-cells found in the distal ileum and colon, is post-translationally processed into GLP-1, a potent insulin secretagogue and other peptides which may mediate small intestinal glucose transport (Holst et al, 1994; Cheeseman & Tsang, 1996). We have recently demonstrated in rats that ingestion of a high fiber diet increases plasma levels of GLP-1(7-37), insulin and C-peptide after oral glucose compared to a fiber-free diet (Reimer & McBurney, 1996). The potent actions of this hormone on carbohydrate metabolism make it a potential candidate in the regulation of glucose homeostasis.

We hypothesized that changes in proglucagon gene expression and postprandial secretion of insulin and C-peptide would differ with the ingestion of physiologic intakes of fibers with different fermentative properties. To test this hypothesis, the effects of a novel source of fiber extracted from rhubarb stalk versus cellulose fiber (5% wt/wt) on proglucagon mRNA, plasma levels of insulin and C-peptide were measured. To determine if fiber type might modulate glucose homeostasis via changes in small intestinal glucose transport, we measured SGLT-1 and GLUT2 mRNA and *in vitro* glucose uptake.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (220-250g) were obtained from the University of Alberta Health Sciences colony. Animals were individually housed in wire mesh bottom cages in a temperature and humidity controlled room with a 12hr light/dark cycle. The protocol was approved by the University of Alberta Animal Welfare Committee.

Animals were given free access to a nonpurified diet (Rodent Laboratory diet PMI #5001, PMI Feeds Inc, St. Louis MO) and water prior to the experimental period. During the experiment animals were given a 5% (wt/wt) fiber diet containing either cellulose or rhubarb stalk fiber and water for ad libitum consumption. Rhubarb fiber was prepared according to procedures described by Basu et al (1993). Composition of the experimental diets is given in **Table 3.1**.

The rats in this study served as controls in a larger study in which diabetes was induced by intravenous penile injection of streptozotocin (20 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) in an acetate buffer (pH 4.5). The animals in this study therefore received a sham injection of the acetate buffer alone on day 3 of the experiment. The animals were continued on their respective diets for an additional 14d.

Food was withheld 16 hr prior to the experiment at which time a tail nick blood sample was taken for fasting plasma glucose and then 50% dextrose by gavage, at a dose of 2g/kg was administered to all rats. At 30 minutes post-gavage animals were anaesthetized and blood taken by cardiac puncture. A 5 cm segment of distal duodenum, jejunum and ileum and proximal colon was excised, flushed with ice cold saline, immersed in liquid nitrogen and stored at -72°C for later mRNA analysis.

Chemicals All chemicals used in Northern Blot analysis and transport kinetic assays were purchased from Sigma Chemical Company (St. Louis, MO), BDH Chemical (Toronto, ON) or Gibco BRL (Burlington, ON). Radioisotopes were purchased from Amersham Canada (Oakville, ON).

Isolation of Total RNA Total RNA was isolated using Trizol™ (Gibco BRL, Burlington, ON, Canada). Isolation was according to the protocol provided with the reagent. RNA was dissolved in DEPC (diethyl pyrocarbonate) treated water and quantity and purity determined by ultraviolet spectrophotometry at 260 and 280 nm.

Northern Blot Analysis Messenger RNA in all samples was measured using a Northern blot analysis procedure described by Fuller et al (1989) with modifications. Aliquots of 15 ug total RNA were dissolved in 10 uL gel loading buffer (50% deionized formamide (vol/vol), 2M formaldehyde, 1.3% glycerol (vol/vol), 0.02 M morpholinopropanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA and 0.1% bromophenol blue (wt/vol)), boiled for 2 min to denature the RNA and then placed on ice for 5 min. Samples were briefly centrifuged and loaded onto 1% agarose (wt/vol) gels containing (0.66M) formaldehyde. RNA was then fractionated according to size by electrophoresis in the presence of a recirculating running buffer containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA (5h at 100V). After electrophoresis, the gels were soaked in two changes of 10X standard saline citrate (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and then blotted onto a MSI Nitropure nitrocellulose membrane (MSI Laboratories, Westboro, MA), employing the capillary method described by Southern (1975). The RNA was then fixed onto membranes by baking in vacuo at 80°C for 2h.

Membranes were prehybridized for 2h at 65°C in prehybridization buffer [6X SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS (wt/vol), 5X Denhart's solution (0.5 g Ficoll 400, 0.5 g PVP, 0.5 g BSA)]. Following prehybridization, membranes were incubated for 16h at 65°C in an identical volume of fresh hybridization buffer with the [^{32}P] ATP-labelled cDNA probe. The 440 bp cDNA proglucagon probe (Taylor et al, 1990), 3.8 kb GLUT2 cDNA probe (donated by Dr. G.I. Bell, Howard Hughes Medical Institute, University of Chicago, IL) and 4.8 kb SGLT-1 cDNA probe (donated by Dr. N. Davidson, University of Chicagon, IL) were labelled by nick translation (Random Primers DNA Labelling System, Life Technologies, Burlington, ON) with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada, Oakville, ON).

Following hybridization, membranes were washed 3 times for 20 min each at room temperature with 2X SSPE, 0.1% (wt/vol) SDS. They were then washed once at 65°C for

20 min with 0.1X SSC, 0.1% (wt/vol) SDS. Membranes were heat sealed in plastic bags and then exposed at -70°C to KODAK XAR5 film (Eastman Kodak, Rochester, NY) using an intensifying screen (Dupont Canada, Mississuaga, ON). For statistical analysis, the signals were quantified using laser densitometry (Model GS-670 Imaging Densitometer, BioRad Laboratories (Canada) Ltd., Mississuaga, ONT). The 28S and 18S ribosomal bands were quantified from negatives of photographs of the membranes. These bands confirm the integrity of the RNA and were used as a denominator for densitometer values to compensate for any loading discrepancies.

Measurement of Transport Kinetics Transport kinetics were determined as previously described (Thomson & Rajotte, 1983). Briefly, the segment (15 cm) of jejunum ileum was opened along its mesenteric border and carefully rinsed with cold saline. Pieces of intestine were cut from the segments and mounted as flat sheets in preincubation chambers containing oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. After 10 minutes the chambers were transferred to other beakers containing [^{3}H]-inulin and various [^{14}C]-probe molecules in oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. The concentration of *D*-glucose was 4, 8, 16, 32 and 64 mM and *L*-glucose was 1 and 16 mM. Uptake of lauric acid (12:0) was used as a measurement of unstirred water layer resistance (13.68 ± 0.62 vs 9.60 ± 1.14 nmol/100 mg/min; cellulose vs rhubarb respectively) . A strobe light was used to adjust stirring rates so that preincubation and incubation stirring rates were identical. To achieve low effective resistance of the intestinal unstirred water layer a stirring rate of 600 rpm was selected (Thomson & Dietschy, 1980). After 6 min in the labelled solution the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for 5 s. A circular steel punch was used to cut the tissue out of the chamber. For all probes the tissue was dried overnight in an oven at 55°C. The dry weight of the tissue was determined, the sample saponified with 0.75 N NaOH, scintillation fluid added (Beckman ReadySolv HP) and radioactivity determined by means of an external standardization technique to correct for variable quenching of the two isotopes. Mucosal weight was determined following

scraping of the intestine from samples of intestine not used for uptake studies. The weight of the mucosa in the samples used to measure uptake was determined by multiplying the dry weight of the intestinal sample by the percentage of the intestinal wall comprised of mucosa. Diet did not alter the proportion of mucosa in the jejunum or ileum, therefore the uptake of nutrients was expressed as nmol*100mg tissue^{-1}*min^{-1}.

Radioimmunoassays Approximately 8 ml of blood were collected from each rat into a chilled syringe of which 6 ml were collected with the addition of EDTA (1 mg/mL blood) and aprotinin (500 KIU/mL blood, Sigma Chemical, St. Louis, MO). The remaining 2 ml were mixed with 80 ul of Heparin Leo (1000 i.u./mL, Leo Laboratories Canada Ltd, Ajax, ON) and 120 ul of NaF (0.025 g/mL, Fisher Scientific). Blood was centrifuged at 1,600 x g for 15 min at 4°C and aliquots taken for glucagon and C-peptide determinations from the EDTA samples. The NaF samples were divided to provide aliquots for insulin and glucose determinations. Samples were stored at -70°C.

C-peptide

Plasma levels of C-peptide were quantified in a single radioimmunoassay using a commercial rat C-peptide RIA kit (Linco Research, St. Louis, MO). The ED_{50} for this assay is 186 pmol/L at a binding of 99.8% (defined as mean total bound minus 2 standard deviations from mean total bound). The intra-assay coefficient of variance was 4.08%.

Insulin and Glucagon

Plasma insulin and glucagon concentrations were measured at the Muttart Diabetes Research Center, University of Alberta. Insulin was determined using a commercial double antibody radioimmunoassay kit (Linco Research, St. Louis, MO) for rat insulin with a detection limit of <2 μU/mL. Plasma levels of glucagon were determined using a commercial double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Plasma Glucose Determination

Plasma glucose was determined using Sigma Diagnostics Glucose (Trinder) Reagent for the enzymatic determination of glucose at 505 nm (Sigma Chemical, St. Louis, MO).

Statistical Analysis Data are given as mean ± SEM. Differences between treatments were determined using the one-way ANOVA model in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Statistical significance is defined as $p \leq 0.05$.

RESULTS

Diet Intake and Growth

Diet intake and weight gain during the experimental period did not differ between groups. Animals consumed 26.1 ± 0.5 and 26.1 ± 0.9 g/d respectively of cellulose and rhubarb diet. Weight gain at 7d was 3.8 ± 0.4 and 4.2 ±0.2 g/d respectively with the cellulose and rhubarb diets. Weight gain at 14d was 6.6 ± 0.3 and 6.5 ± 0.3 g/d respectively on cellulose and rhubarb diets. Dietary fiber source did not affect total weight of jejunum, ileum and colon or % mucosa in the jejunum and ileum **(Table 3.2)**.

Blood Parameters

Postprandial C-peptide levels in rats consuming the rhubarb fiber diet were significantly higher than those consuming the cellulose fiber diet **(Figure 3.1A)**. Plasma insulin tended to be higher with rhubarb fiber 30 minutes after oral glucose but the increase was not significant **(Figure 3.1B)**. Consumption of cellulose versus rhubarb fiber did not alter levels of plasma glucagon (93.6 ± 5.1 vs 98.4 ± 3.3 ng/L), fasting glucose (97.1 ± 5.3 vs 99.5 ± 5.1 mg/dL) and 30 minute glucose (162.0 ± 4.5 ± 156 ± 6.3 mg/dL).

mRNA Abundance

The 440 bp proglucagon mRNA fragment was readily detected in total RNA from ileum and colon. Mean densitometric readings for proglucagon mRNA were significantly

higher in the ileum ($p<0.05$) with rhubarb fiber **(Figure 3.2)** but remained unaffected in the colon **(Figure 3.3)**. Fiber type did not affect the expression of the brush border glucose transporter, SGLT-1 or the basolateral glucose transporter, GLUT2 **(Figure 3.4A & 3.4B)**.

In Vitro Nutrient Uptake

The apparent passive permeability coefficient, estimated with L-glucose, was significantly higher ($p<0.05$) at both 1 mM and 16 mM concentrations in the jejunum of the animals fed the cellulose versus rhubarb fiber diet **(Table 3)**. Fructose uptake in the jejunum and ileum, however, did not differ between the fiber diets **(Table 3)**. Mean values of the maximum transport rates (Vmax) **(Table 4)** were not altered with diet. Estimated values for the apparent Michaelis affinity constant (Km) were significantly higher in the jejunum of animals fed cellulose versus rhubarb fiber diet ($p<0.05$) **(Table 4)**.

DISCUSSION

We have previously shown that feeding a high fiber diet upregulates proglucagon mRNA compared to a fiber free elemental diet (Reimer & McBurney, 1996). In this study the level of fiber was similar in both diets. We now report that ingestion of a more fermentable dietary fiber, rhubarb fiber, at 5% of the diet (wt/wt) is associated with increased ileal proglucagon mRNA and postprandial C-peptide concentrations compared to cellulose.

These results suggest that fiber type in addition to the level of fiber intake may be an important factor in the regulation of proglucagon gene expression. Supplementing total parenteral nutrition (TPN) with SCFA significantly increases proglucagon mRNA abundance in rats after massive small bowel resection (Tappenden et al, 1996). Moreover, plasma concentrations of enteroglucagon have been shown to be increased

with the ingestion of fermentable dietary fiber (Southon et al, 1987; Gee et al, 1996). Under *in vitro* fermentation conditions, rhubarb stalk and cellulose produce 6.5 mmol/g and 2.5 mmol SCFA/g, respectively (unpublished data). Evidence therefore suggests that SCFA's resulting from the fermentation of dietary fiber in the colon may be key players in the modulation of proglucagon gene expression seen with dietary fiber.

Nutrient movement across the intestinal epithelium occurs via passive permeation or carrier-mediated uptake. Tight junctions (paracellular pathway) and the enterocyte (transcellular pathway) mediate passive permeation (Ballard et al, 1995; Karasov & Diamond, 1983). In this study, cellulose increased the permeability of the paracellular pathway in the jejunum, suggesting that fiber type altered nutrient delivery per se or intestinal permeability. The physiological significance of the paracellular pathway has been questioned (Fedorak et al,1990), however, because only during periods of high intraluminal glucose concentrations may the paracellular pathway comprise up to 30% of total absorbed glucose (Pappenheimer & Reiss, 1987). Despite playing a modest role in overall glucose uptake, the increased permeability of the jejunum seems to indicate that the jejunum of animals fed cellulose may be more 'leaky' than that of the rhubarb fiber fed animals. The fact that L-glucose uptake accounts for approximately 25% of total glucose uptake in this study indicates that this route of glucose absorption may be important in certain states. Although controversial, the lower passive permeability seen with rhubarb suggests that overall glucose absorption may be reduced with rhubarb fiber.

The lack of change in the expression of the glucose transporters, SGLT-1 and GLUT2 is reflected in the similar values for the maximum transport rates (Vmax) between diet groups. Protein mediated transport is predominantly altered by changes in Vmax (Karasov & Diamond, 1983). The quantity or activity of transporters was therefore not altered with ingestion of the two fiber types examined in this study. Changes did, however, occur in the apparent Michaelis affinity constant (Km). Changes in Km may reflect changes in the unstirred water layer resistance (Urrutia et al, 1988). Interestingly,

unstirred water layer resistance as measured by uptake of fatty acid 12:0 was significantly greater in the ileum with rhubarb fiber but not in the jejunum. Indeed, Flourie et al (1984) suggested that pectin, a viscous fiber, may delay absorption of glucose by increasing the thickness of the unstirred water layer. Even though GLUT5 mRNA was not measured, the lack of change in fructose uptake lends support to the role changes in unstirred water layer resistance may play in altering passive permeability. Rhubarb fiber in contrast to cellulose results in higher SCFA production which may exert systemic effects which alter conformation of the transporter. Evidence to support this action has not been examined in the literature.

The lower Km observed in this study suggests that when luminal concentrations of glucose are low, glucose carriers would have a higher affinity for glucose with rhubarb compared to cellulose. Early after a meal changes in Vmax are largely responsible for determining glucose uptake. As luminal glucose concentrations fall with increased duration after a meal, changes in Km become important. Indeed, the higher affinity observed with rhubarb may partially be compensated for by the reduced rate of gastric emptying seen with elevations in plasma GLP-1. Willms et al (1996) have recently demonstrated that the potent actions of GLP-1 on glucose homeostasis may in large part be due to the significant reduction in gastric emptying seen with GLP-1.

More fermentable dietary fibers appear to enhance glucose homeostasis via several mechanisms. Firstly, the increase in proglucagon mRNA and more specifically the secretion of the potent insulin secretagogue, GLP-1 enhance the disposition of absorbed glucose in peripheral tissues. Earlier work demonstrates a significant enhancement of GLP-1 secretion with high fiber feeding (Reimer & McBurney, 1996). C-peptide measurements provide a better estimate of insulin secretory rate than peripheral insulin measurements (Polonsky & Rubenstein, 1984) and higher concentrations of C-peptide with the more fermentable rhubarb fiber therefore suggest an actual enhancement of insulin secretion in this study.

Secondly, it is well established that viscous fibers delay diffusion of glucose from dialysis bags whereas particulate fibers have little effect (Jenkins et al, 1980; Jenkins et al, 1984). This action is largely due to the ability of more soluble fibers to increase the viscosity of glucose solutions *in vitro* and luminal contents *in vivo*. Total glucose uptake was not altered with the experimental diets in this study but the lower Km seen with the rhubarb fiber may reflect an increase in unstirred water layer resistance, theoretically slowing the rate of glucose absorption. Many studies have shown long term ingestion of fiber improves glucose tolerance (Groop et al, 1993; Pastors et al, 1991). Until recently it was thought that the physical properties of fibers were solely responsible for improvements in glucose homeostasis. Evidence now suggests that gastrointestinal hormones may play an important role as well. We suggest that these mechanisms work in concert to improve overall glucose homeostasis seen with fermentable fibers.

It is clear that the small intestine is able to alter nutrient absorptive capacity in response to many physiological and pathological states including dietary changes, pregnancy and lactation, intestinal resection and in the disease states of diabetes and starvation (Reviewed by Philpott, 1992). The precise signals for the regulation of nutrients are as yet incompletely understood. In this study we have demonstrated that type of dietary fiber is important in the regulation of insulinotropic intestinal hormone expression. Specifically rhubarb fiber resulted in an upregulation of ileal proglucagon mRNA and enhanced C-peptide secretion. Alterations in passive permeability and Km support the role of more fermentable, viscous fibers in delaying the rate of glucose absorption.

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TABLE 3.1 Composition of the Experimental Diets

Ingredient	Cellulose	Rhubarb
	g/kg diet	
Starch	648	648
Casein	200	200
Vitamin Mix[1]	10	10
Mineral Mix[1]	30	30
Corn Oil	60	60
Methionine	2	2
Fiber[2]	50	50

[1] Supplied in quantities adequate to meet NRC requirements.

[2] Cellulose was supplied as Alphacel non-nutritive bulk (ICN Biomedicals Inc., Aurora, OH). Rhubarb stalk fiber was prepared in Dr. B. Ooarikul's laboratory at the University of Alberta.

TABLE 3.2 Effect of Dietary Fiber on Intestinal Characteristics

	Diet		
	Cellulose	**Rhubarb**	**Difference Between Diets**
Jejunum			
Total Weight (mg/cm)	10.9 ± 0.94	13.2 ± 2.07	NS
% Mucosa	39.3 ± 4.30	43.3 ± 6.96	NS
Ileum			
Total Weight (mg/cm)	14.1 ± 2.07	14.0 ± 1.87	NS
% Mucosa	43.4 ± 7.89	53.6 ± 4.11	NS
Colon			
Total Weight (g)	0.88 ± 0.03	0.83 ± 0.05	NS

Values are mean ± SEM (n=6 rats / treatment). NS = Not significant

TABLE 3.3 Effect of Dietary Fiber on Intestinal L-Glucose and Fructose Uptake

	Diet		
	Cellulose	**Rhubarb**	**Difference Between Diets**
L-Glucose			
Jejunum			
16 mM	202.76 ± 16.99	122.81 ± 15.35	$p<0.05$
Ileum			
16 mM	168.08 ± 18.79	137.41 ± 16.31	NS
Fructose			
Jejunum	10.42 ± 0.18	10.93 ± 0.64	NS
Ileum	12.33 ± 0.85	13.43 ± 0.79	NS

Values are mean ± SEM (n=6 rats / treatment). Units of uptake are nmol/100 mg tissue /minute. NS = Not significant.

TABLE 3.4 Effect of Dietary Fiber on D-glucose Uptake Kinetics in Rats (corrected for passive permeability and unstirred water layer resistance)

	Cellulose Fiber Diet		Rhubarb Fiber Diet	
	Vmax	**Km**	**Vmax**	**Km**
Jejunum	544 ± 119	16.9 ± 7.4	499 ± 50	1.1 ± 0.4*
Ileum	333 ± 102	13.2 ± 6.8	359 ± 41	0.6 ± 0.3

Values are mean ± SEM (n=6 rats / treatment). Units for Vmax are nmol/100 mg tissue/min. Units for Km are mM. The symbol '*' represents a significant difference between rhubarb and cellulose fiber ($p<0.05$).

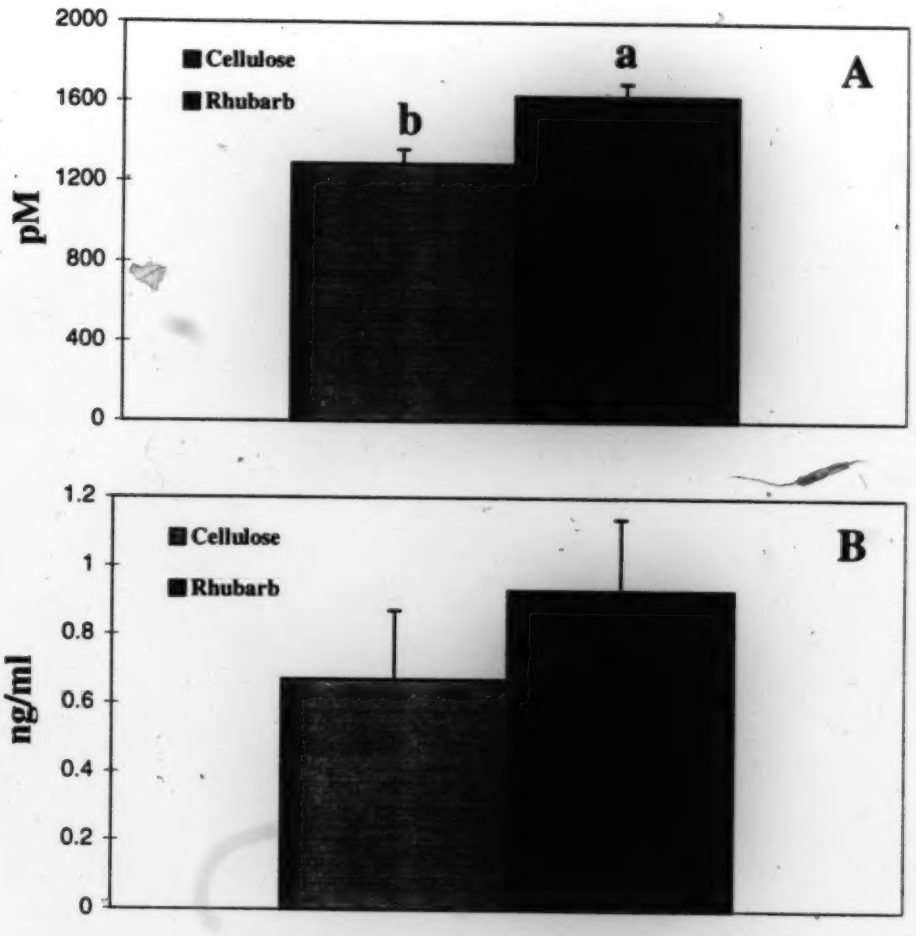

Figure 3.1 Plasma levels of C-peptide (A) and insulin (B) 30 minutes post-oral glucose load in rats fed either rhubarb or cellulose fiber diet. Values are mean ± SEM (n=10 rats / treatment). Values with different letters are significantly different ($p<0.05$).

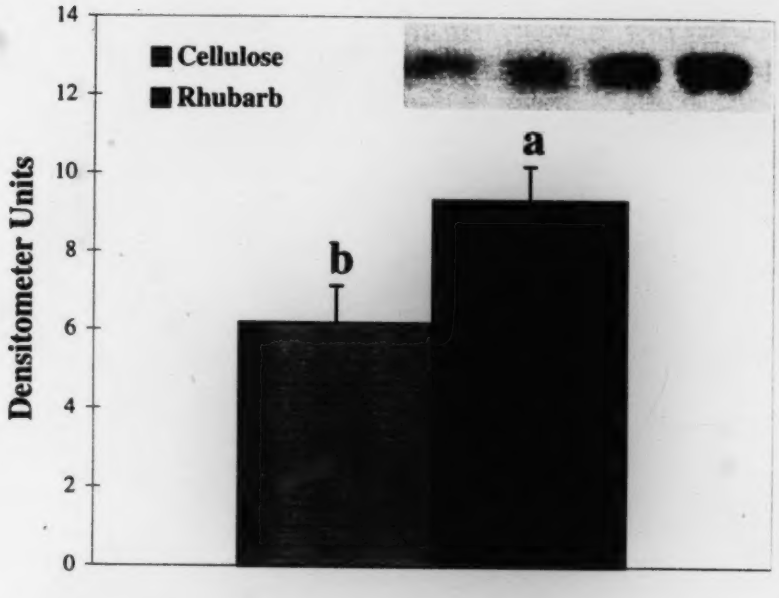

Figure 3.2 Effect of rhubarb and cellulose fiber on ileal proglucagon mRNA expression in rats. Values are mean ± SEM (n=8 rats / treatment). Values with different letters are significantly different ($p<0.05$). Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right in duplicate from two individual rats: Cellulose, Rhubarb.

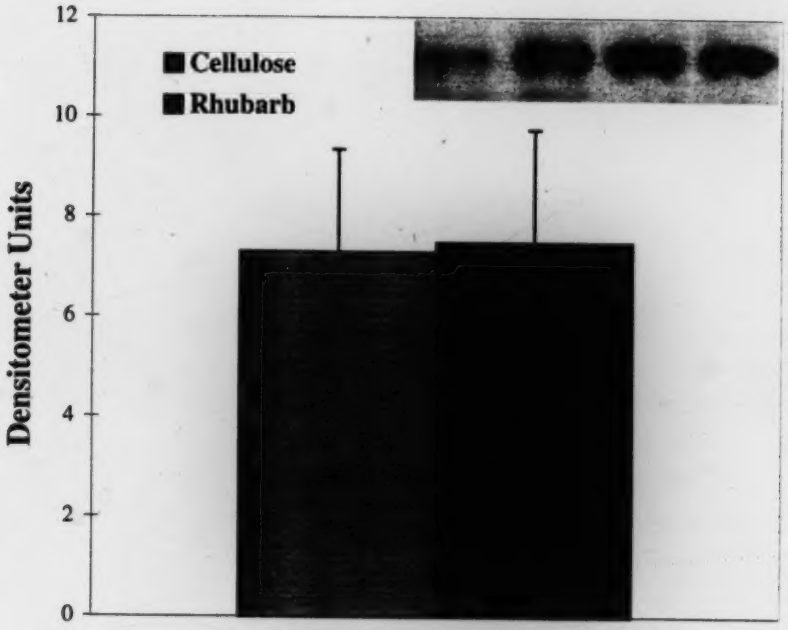

Figure 3.3 Effect of rhubarb and cellulose fiber on colonic proglucagon mRNA expression in rats. Values are mean ± SEM (n=8 rats / treatment). Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right in duplicate from two individual rats: Cellulose, Rhubarb.

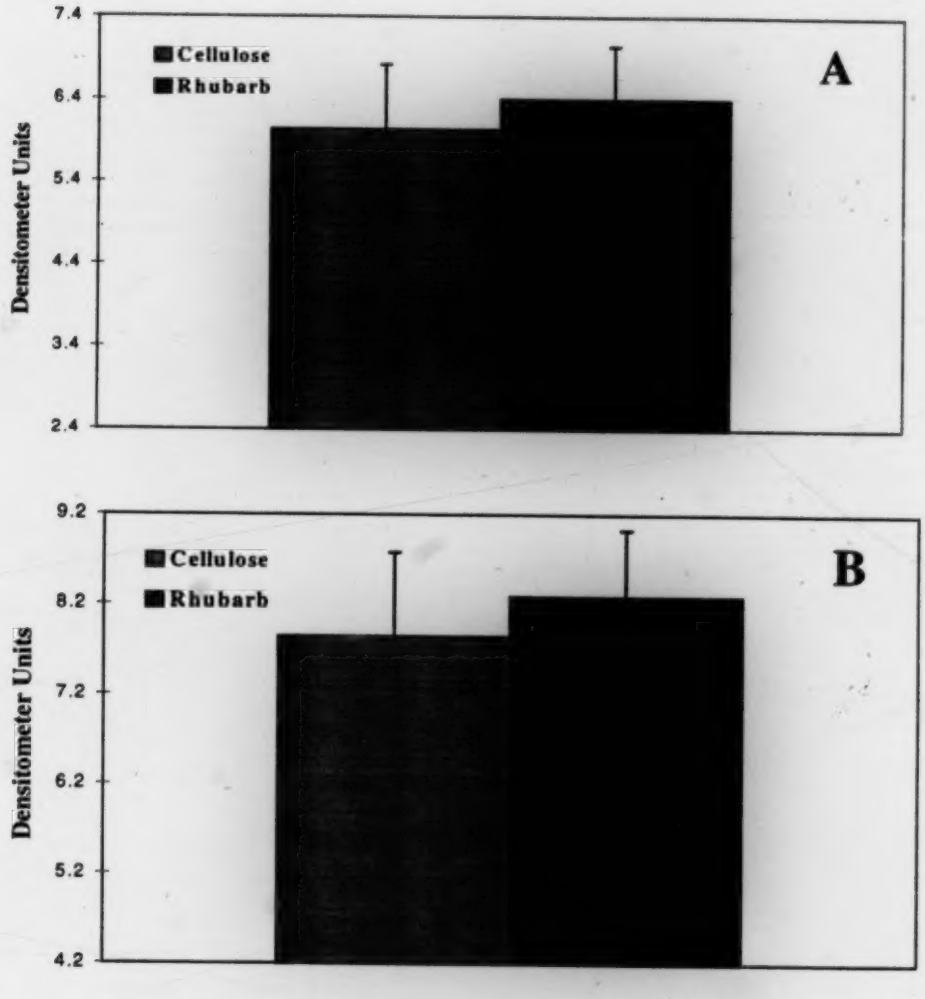

Figure 3.4 Effect of rhubarb and cellulose fiber on jejunal SGLT-1 (A) and GLUT2 (B) mRNA expression in rats. Values are mean ± SEM (n=8 rats / treatment). No significant differences between diets were found.

CHAPTER 4

RHUBARB FIBER AND FOOD INTAKE REGULATE PROGLUCAGON GENE EXPRESSION AND INTESTINAL NUTRIENT UPTAKE IN DIABETIC RATS[1]

INTRODUCTION

Endocrine cells found in the distal ileum and colon produce the proglucagon-derived peptides, enteroglucagon (glicentin and oxyntomodulin), GLP-1 and GLP-2 (Mojsov et al, 1986). GLP-2 has been shown to enhance jejunal glucose uptake in rats (Cheeseman & Tsang, 1996). GLP-1 slows gastric emptying rate (Willms et al, 1996) and stimulates insulin secretion from pancreatic β-cells (Holst, 1994). Exogenous GLP-1 has been proposed to stimulate insulin secretion and improve glucose management in non-insulin dependent diabetic subjects (Nauck, 1996; Nauck et al, 1996).

We have previously demonstrated that ingestion of fermentable fiber increases intestinal proglucagon expression and the secretion of GLP-1 and insulin in response to oral glucose in normal animals (Reimer & McBurney, 1996, Reimer et al, submitted). It remains to be elucidated if diabetic animals respond to fermentable dietary fiber with increased proglucagon mRNA and GLP-1.

The current study was undertaken to assess the effect of fibers differing in fermentative properties and food intake on intestinal proglucagon mRNA and plasma concentrations of GLP-1, insulin, and c-peptide after oral glucose in streptozotocin-diabetic animals. *In vitro* intestinal glucose uptake and glucose transporter (SGLT-1 and GLUT2) mRNA abundance were also measured to assess the effect of diet on intestinal glucose absorptive

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capacity. Because relatively little is known regarding intestinal function in the BB rat, we examined this model of autoimmune-associated insulin dependent diabetes mellitus as well. Thus, the primary objective of this study was to determine the effect of fiber fermentability and food intake on intestinal proglucagon expression. It is our hypothesis that proglucagon-derived peptides modulate glucose absorptive capacity and insulin secretion to influence glucose homeostasis.

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (220-250g) were obtained from the University of Alberta Health Sciences colony. Male diabetic-prone (BBd) rats (310-400g) were obtained from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, ON, Canada). BBdp rats were diabetic upon arrival from Health Canada and were maintained with insulin implants (Linplant™, Scarborough, ON, Canada) delivering 2U/d. Animals were individually housed in wire mesh bottom cages in a temperature and humidity controlled room with a 12hr light/dark cycle. The protocol was approved by the University of Alberta Animal Welfare Committee.

Animals were given a nonpurified diet (Rodent Laboratory diet PMI #5001, PMI Feeds Inc, St. Louis MO) and water for ad libitum consumption prior to the experimental period. During the experiment animals consumed either a 5% (wt/wt) cellulose or 5% (wt/wt) rhubarb stalk fiber diet. Animals had free access to the diet during the experimental period. One group of streptozotocin-diabetic animals were restrict-fed at a level previously determined to be consumed by normal, non-diabetic animals of similar age and weight (Reimer et al, submitted). Composition of the experimental diets is given in **Table 4.1**.

After the rats had acclimatized to the metabolic cages and consumed the diet for 3d, diabetes was induced in the Sprague-Dawley rats by intravenous penile injection of streptozotocin (20 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) in an acetate

buffer (ph 4.5) and the BBd rats received a sham injection of the acetate buffer alone. The animals were continued on their respective diets for an additional 14d. On the day prior to sacrifice (1500hr) insulin implants were removed from the BBd rats to prevent hypoglycemia during the overnight fast.

After an overnight fast, a tail nick blood sample was taken for fasting plasma glucose and then 50% dextrose at a dose of 2g/kg was administered by gavage to all rats. At 30 minutes post-gavage animals were anesthetized and blood taken by cardiac puncture. A 5 cm segment of distal duodenum, jejunum and ileum and proximal colon was excised, flushed with ice cold saline, immersed in liquid nitrogen and stored at -72 C for later mRNA analysis.

Isolation of Total RNA Total RNA was isolated using Trizol™ (Gibco BRL, Burlington, ON, Canada). Isolation was according to the protocol provided with the reagent. RNA was dissolved in DEPC (diethyl pyrocarbonate) treated water and quantity and purity determined by ultraviolet spectrophotometry at 260 and 280 nm.

Northern Blot Analysis Messenger RNA in all samples was measured using a Northern blot analysis procedure described by Fuller et al (1989) with modifications. Aliquots of 15 ug total RNA were dissolved in 10 uL gel loading buffer (50% deionized formamide (vol/vol), 2M formaldehyde, 1.3% glycerol (vol/vol), 0.02 M morpholinopropanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA and 0.1% bromophenol blue (wt/vol)), boiled for 2 min to denature the RNA and then placed on ice for 5 min. Samples were centrifuged briefly and loaded onto 1% agarose (wt/vol) gels containing (0.66M) formaldehyde. RNA was then fractionated according to size by electrophoresis in the presence of a recirculating running buffer containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA (5h at 100V). After electrophoresis, the gels were soaked in two changes of 10X standard saline citrate (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and then blotted onto a MSI Nitropure nitrocellulose

membrane (MSI Laboratories, Westboro, MA), employing the capillary method described by Southern (1975). The RNA was then fixed onto membranes by baking in vacuo at 80 C for 2h.

Prior to hybridization with the [^{32}P] ATP-labeled cDNA probe, membranes were prehybridized for 2h at 65 C in prehybridization buffer [6X SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS (wt/vol), 5X Denhart's solution (0.5 g Ficoll 400, 0.5 g PVP, 0.5 g BSA (frakcia v)]. Following prehybridization, membranes were incubated for 16h at 65 C in an identical volume of fresh hybridization buffer with the addition of labeled cDNA probe. The 440 bp cDNA proglucagon probe (Taylor et al, 1990), 3.8 kb GLUT2 cDNA probe (donated by Dr. G.I. Bell, Howard Hughes Medical Institute, University of Chicago, IL) and 4.8 kb SGLT-1 cDNA probe (donated by Dr. N. Davidson, University of Chicago, IL) were labelled by nick translation (Random Primers DNA Labelling System, Life Technologies, Burlington, ON) with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada, Oakville, ON).

Following hybridization, membranes were washed 3 times for 20 min each at room temperature with 2X SSPE, 0.1% (wt/vol) SDS. They were then washed once at 65 C for 20 min with 0.1X SSC, 0.1% (wt/vol) SDS. Membranes were heat sealed in plastic bags and then exposed at -70 C to KODAK XAR5 film (Eastman Kodak, Rochester, NY) using an intensifying screen (Dupont Canada, Mississauga, ON). For statistical analysis, the signals were quantified using laser densitometry (Model GS-670 Imaging Densitometer, BioRad Laboratories (Canada) Ltd., Mississauga, ONT). The 28S and 18S ribosomal bands were quantified from negatives of photographs of the membranes. These bands confirm the integrity of the RNA and were used as a denominator for densitometer values to compensate for any loading discrepancies.

Radioimmunoassays Approximately 8 ml of blood were collected from each rat into a chilled syringe. Approximately 6 ml of blood were collected with the addition of EDTA

(1 mg/ml blood) and aprotinin (500 KIU/ml blood, Sigma Chemical, St. Louis, MO). The remaining 2 ml were mixed with 80 ul of Heparin Leo (1000 i.u./ml, Leo Laboratories Canada Ltd, Ajax, ON) and 120 ul of NaF (0.025 g/ml, Fisher Scientific). Blood was centrifuged at 1,600 x g for 15 min at 0 C and aliquots taken for GLP-1, glucagon and C-peptide determinations from the EDTA samples. The NaF samples were divided to provide aliquots for insulin and glucose determinations. Samples were stored at -70 C.

GLP-1(7-36) Amide

GLP immunoreactive peptides were extracted from 2.5 ml of plasma using a SEP-COLUMN containing 200 mg of C_{18} (Cat # RIK-SEPCOL 1, Peninsula Laboratories, Belmont, CA) with Buffer A (0.1% trifluoroacetic acid (Peninsula Laboratories, Belmont, CA)) and Buffer B (60% acetonitrile (Peninsula Laboratories, Belmont, CA)) as elution solvents. The extraction was performed according to the protocol provided with the GLP-1-(7-37) RIA kit. The recovery rate of the extracted peptide was 50% using this method. Concentrations of GLP-1(7-36) amide were measured using a double-antibody radioimmunoassay kit (Peninsula Laboratories, Belmont, CA). This kit measures GLP-1(7-36) amide with 0.4% crossreactivity with GLP-1-(7-37). The intra-assay coefficient of variance was 1.90%.

C-Peptide

Plasma levels of C-peptide were quantified in a single radioimmunoassay using a commercial rat c-peptide RIA kit (Linco Research, St. Louis, MO). The ED_{50} for this assay is 397 pmol/L at a binding of 99.8% (defined as mean total bound minus 2 standard deviations from mean total bound). The intra-assay coefficient of variance was 3.65%.

Insulin and Glucagon

Plasma insulin and glucagon concentrations were measured at the Muttart Diabetes Research Center, University of Alberta. Insulin was determined using a commercial

double antibody radioimmunoassay kit (Linco Research, St. Louis, MO) for rat insulin with a detection limit of <2 uU/ml. Plasma levels of glucagon were determined using a commercial double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Plasma Glucose Determination

Plasma glucose was determined using Sigma Diagnostics Glucose (Trinder) Reagent for the enzymatic determination of glucose at 505 nm (Sigma Chemical, St. Louis, MO).

Measurement of Transport Kinetics Transport kinetics were determined as previously described (Thomson & Rajotte, 1983). Briefly, the segment (15 cm) of jejunum ileum was opened along its mesenteric border and carefully rinsed with cold saline. Pieces of intestine were cut from the segments and mounted as flat sheets in preincubation chambers containing oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. After 10 minutes the chambers were transferred to other beakers containing [^{3}H]-inulin and various [^{14}C]-probe molecules in oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37°C. The concentration of *D*-glucose was 4, 8, 16, 32 and 64 mM and *L*-glucose was 1 and 16 mM. Uptake of lauric acid (12:0) was used as a measurement of unstirred water layer resistance. A strobe light was used to adjust stirring rates so that preincubation and incubation stirring rates were identical. To achieve low effective resistance of the intestinal unstirred water layer a stirring rate of 600 rpm was selected (Thomson & Dietschy, 1980). After 6 min in the labelled solution the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for 5 s. A circular steel punch was used to cut the tissue out of the chamber. For all probes the tissue was dried overnight in an oven at 55°C. The dry weight of the tissue was determined, the sample saponified with 0.75 N NaOH, scintillation fluid added (Beckman ReadySolv HP) and radioactivity determined by means of an external standardization technique to correct for variable quenching of the two isotopes. Mucosal weight was determined following scraping of the intestine from samples of intestine not used for uptake studies. The

weight of the mucosa in the samples used to measure uptake was determined by multiplying the dry weight of the intestinal sample by the percentage of the intestinal wall comprised of mucosa. Diet did not alter the proportion of mucosa in the jejunum or ileum, therefore the uptake of nutrients was expressed as nmol*100mg tissue^{-1}*min^{-1}.

Statistical Analysis Data are given as mean ± SEM. Data was analyzed using orthogonal contrasts in SAS (Version 6.04, SAS Institute, Cary, NC) where *t*-1 comparisons can be made to determine information concerning the population means. Nutrient uptake measurements were estimated according to Churnratanakul et al (1990) using a commercially available statistical program, Systat (SYSTAT, Inc., Illinois), employing an iterative, non-linear regression method. As the variance is decreased by weighting individual data points to generate a single curve per treatment, the use of orthogonal contrasts for this set of data is not possible. Predetermined comparisons were made using paired t-tests between disease states including streptozotocin-diabetic ad libitum fed vs. streptozotocin-diabetic restrict animals and streptozotocin-diabetic ad libitum fed vs. ad libitum fed BBd animals. Statistical significance is defined as $p \leq 0.05$.

RESULTS

Food Intake and Growth

Food intake was unaffected by diet (**Table 4.2**). Feed restriction of streptozotocin-diabetic animals did not significantly affect 7 or 14d weight gains versus ad libitum fed streptozotocin-diabetic animals. At 14d streptozotocin-diabetic ad libitum fed animals gained significantly more weight than the BBd animals.

Intestinal Characteristics

Jejunal, ileal and colonic weights were significantly affected by food intake (**Table 4.3**). The weight of these segments was also significantly different in autoimmune (BBd) versus streptozotocin-diabetic animals. No significant comparisons were found for % mucosa in the jejunum or ileum.

Proglucagon, SGLT-1 and GLUT2 mRNA

Consumption of rhubarb versus cellulose fiber resulted in significant differences for ileal and colonic proglucagon and jejunal SGLT-1 mRNA abundance **(Figure 4.1-4.3)**. Comparison of rhubarb versus cellulose fiber, strictly in ad libitum fed animals (BBd and Streptozotocin) was significant. A significant effect of food intake (ad libitum versus restrict fed) was seen for jejunal SGLT-1 and GLUT2 mRNA **(Figure 4.3 and 4.4)**. Comparison of food intake between streptozotocin ad libitum and restrict fed animals was significant for levels of SGLT-1 but not GLUT2 mRNA. Significant differences due to diet were seen in abundance of SGLT-1 mRNA. Changes in SGLT-1 and GLUT2 mRNA were significantly different in autoimmune versus streptozotocin-diabetes.

Blood Parameters

A significant effect of food intake (ad libitum versus restrict fed) was observed for fasting plasma glucose and plasma glucose, insulin, c-peptide, and glucagon levels 30 minutes post-oral glucose **(Table 4.5)**. Restrict feeding streptozotocin-diabetic animals resulted in differences in fasting plasma glucose and plasma glucose and c-peptide 30 minutes post-oral glucose. Significant differences due to diet were seen in plasma levels of insulin, c-peptide and GLP-1(7-36) amide. For all parameters, except GLP-1(7-36) amide, autoimmune diabetic animals were significantly different than streptozotocin-diabetic animals.

In Vitro Glucose Uptake

Passive permeability as measured by L-glucose and D-fructose uptake was significantly higher in the ileum of restrict-fed streptozotocin-diabetic animals consuming cellulose versus rhubarb **(Table 4.5)**. In both diet groups, ileal uptake of L-glucose was significantly higher in restrict-fed versus ad libitum fed streptozotocin-diabetic animals. Fructose uptake was higher in the jejunum and lower in the ileum of restrict-fed versus ad libitum fed streptozotocin-diabetic animals consuming rhubarb fiber. Restrict-fed animals consuming rhubarb had higher jejunal uptake of fructose than ad libitum fed

streptozotocin-diabetic animals consuming rhubarb. BBd consuming cellulose had higher ileal fructose uptake than ad libitum fed streptozotocin-diabetic animals consuming cellulose.

Ad libitum consumption of rhubarb fiber by streptozotocin-diabetic animals resulted in significantly higher ileal Vmax than cellulose fiber (**Table 4.6**). In both diet groups, ad libitum fed streptozotocin-diabetic animals had significantly higher jejunal Vmax than BBd animals. Rhubarb fiber increased jejunal Km in BBd animals and increased ileal Km in ad libitum fed streptozotocin-diabetic animals. Ad libitum fed streptozotocin-diabetic animals consuming cellulose had significantly higher jejunal Km than BBd consuming cellulose.

DISCUSSION

The ingestion of dietary fiber reduces exogenous insulin requirements, lowers fasting and postprandial plasma glucose concentrations and improves glycemic control in diabetic patients (Jenkins et al, 1978; Vinik & Jenkins, 1988; Pastors et al, 1991). Soluble fibers are known to increase the viscosity of luminal contents and slow glucose absorption (Jenkins et al, 1978; Holt et al, 1979). We have suggested that the ingestion of fermentable fiber increases intestinal proglucagon expression and GLP-1 secretion to influence glucose homeostasis in normals animals (Reimer et al, 1996; Reimer et al, submitted). However, we now report that proglucagon mRNA abundance does not increase in diabetic animals consuming a more fermentable fiber source, rhubarb. Nevertheless, plasma levels of GLP-1(7-36) amide were found to be higher 30 minutes after oral glucose in diabetic rats fed rhubarb although this response achieved significance in the BBd rat. The BBd rat is insulinopenic and cannot respond to the insulinotropic actions of GLP-1.

Glucose homeostasis is a function of glucose entry rate and glucose disposal. Willms et

al (1996) suggested that GLP-1 may inhibit gastric emptying rate and delay glucose absorption. Conversely, GLP-2 is also derived from proglucagon and seems to upregulate glucose transporter abundance (Cheeseman & Tsang, 1996). Although controversial, it has been suggested that up to 30% of glucose absorption may occur via the paracellular (tight junction) pathway (Pappenheimer & Reiss, 1987). Passive permeability was affected by amount of food intake with increased 'leakiness' associated with lower food intake and cellulose fiber. Indeed, L-glucose uptake accounts for approximately 25% of total glucose uptake in this study and indicates that this passive route of glucose absorption may be important in certain states.

Vmax was reduced with rhubarb and lower in BBd versus streptozotocin-diabetic rats. Similarly, Km was significantly influenced by food intake but decreased with rhubarb fiber and was lower in BBd animals. These functional measurements were not reflected in mRNA abundance of jejunal glucose transporters. Jejunal glucose transporter abundance was influenced by food intake with ad libitum fed animals having lower mRNA abundance. Fermentable fiber did not affect GLUT2 mRNA although SGLT-1 was increased with rhubarb. Expression of intestinal glucose transporters may be dissociated from functional uptake in the hypertrophic diabetic intestine.

In conclusion, several studies have shown that intestinal mass and nutrient transporters are regulated differently in diabetic versus normal animals, independent of hyperphagia (Fedorak et al, 1990). In this study food intake or amount of food consumed significantly affected many parameters including plasma glucose, insulin, c-peptide and glucagon as well as the intestinal glucose transporters, SGLT-1 and GLUT2 mRNA. Proglucagon mRNA and plasma GLP-1(7-36) amide were significantly affected by the ingestion of fermentable fiber but not food intake per se. This suggests that proglucagon abundance is regulated by fermentation end-products, whereas SGLT-1 and GLUT2 mRNA and plasma levels of glucose and insulin also respond to the delivery of nutrients into the small intestine per se. Indeed, Tappenden et al, 1996 demonstrated that intravenous

SCFA upregulated proglucagon mRNA and increased functional glucose uptakes and GLUT2, SGLT-1 and Na^+-K^+ ATPase in the residual ileum of parenterally fed rats after intestinal surgery.

The direct positive relationship between intestinal proglucagon expression and GLP-1 secretion in normal animals (Reimer et al, 1996; Reimer et al, submitted) does not extend to the diabetic state. For whatever reason, despite lower proglucagon mRNA in diabetic rats fed a fermentable fiber, increased concentrations of GLP-1 are found in the plasma 30 minutes after an oral glucose load. Gastric inhibitory polypeptide (GIP) release seems to be dependent upon intestinal glucose absorption rates (Ebert & Creutzfeldt, 1980; Schirra et al, 1996). Perhaps the greater glucose transport observed in the jejunum and ileum of rhubarb fed rats serves to signal GLP-1 release. The relationship between diet, diabetes and the synthesis of proglucagon-derived peptides deserves further investigation.

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TABLE 4.1 Composition of the Experimental Diets

Ingredient	Cellulose	Rhubarb
	g/kg diet	
Starch	648	648
Casein	200	200
Vitamin Mix[1]	10	10
Mineral Mix[1]	30	30
Corn Oil	60	60
Methionine	2	2
Fiber[2]	50	50

[1] Supplied in quantities adequate to meet NRC requirements.

[2] Cellulose was supplied as Alphacel non-nutritive bulk (ICN Biomedicals Inc., Aurora, OH). Rhubarb stalk fiber was prepared in Dr. B. Ooarikul's laboratory at the University of Alberta.

TABLE 4.2 Diet Intake and Weight Gain

	Diet		
	Cellulose	**Rhubarb**	**Difference Between Diets**
Feed Intake (g/d)			
BBd	33.9 ± 2.5	34.7 ± 2.4	NS
Streptozoticin Diabetic	35.1 ± 1.8[a]	33.5 ± 1.2[a]	NS
Restrict-Fed Strep Diabetic	24.6 ± 0.5[b]	24.7 ± 0.7[b]	NS
Weight gain at 7 d (g/d)			
BBd	0.7 ± 0.4	1.1 ± 0.4	NS
Streptozoticin Diabetic	1.0 ± 0.4	0.9 ± 0.2	NS
Restrict-Fed Strep Diabetic	0.3 ± 0.3	0.7 ± 0.3	NS
Weight gain at 14 d (g/d)			
BBd	0 ± 0.6[a]	0 ± 0.6[a]	NS
Streptozoticin Diabetic	2.4 ± 0.6[b]	1.7 ± 0.5[b]	NS
Restrict-Fed Strep Diabetic	1.1 ± 0.4	1.7 ± 0.3	NS

Values are mean ± SEM (n=10 rats / treatment). NS = Non-significant. Superscripts 'a' and 'b' represent a significant difference ($p<0.05$) between disease state. Comparisons are between BBd and streptozotocin-diabetic ad libitum fed animals and between streptozotocin-diabetic ad libitum and restrict fed animals. Weight gain represents a change in weight from day 0.

TABLE 4.3 Effect of Dietary Fiber on Intestinal Characteristics in Diabetic Rats

	Cellulose Fiber Diet			Rhubarb Fiber Diet		
	BBd	**Induced Diabetic**	**Restrict-Fed**	**BBd**	**Induced Diabetic**	**Restrict-Fed**
Jejunum						
Total Weight (mg/cm)	15.6 ± 0.91	15.1 ± 1.40	8.9 ± 0.74	16.9 ± 0.83	13.9 ± 1.21	12.4 ± 0.91
% Mucosa	45.5 ± 5.35	51.1 ± 6.83	41.8 ± 4.90	49.0 ± 7.17	58.5 ± 5.56	52.8 ± 3.11
Ileum						
Total Weight (mg/cm)	18.3 ± 0.59	13.5 ± 2.04	11.7 ± 0.65	21.8 ± 1.89	16.0 ± 1.14	11.3 ± 0.92
% Mucosa	45.0 ± 2.21	43.2 ± 5.53	40.2 ± 3.70	46.5 ± 5.85	52.3 ± 4.39	46.1 ± 6.57
Colon						
Total Weight (g)	1.36 ± 0.10	0.89 ± 0.07	0.91 ± 0.06	1.40 ± 0.08	1.10 ± 0.09	0.97 ± 0.05

Values are mean ± SEM (n=6 rats / treatment). Statistics are described in the chart of orthogonal contrasts.

Orthogonal Contrasts for Intestinal Characteristics (Table 4.3)

Parameter	Source	df	P
Jejunum Weight	Ad libitum vs restrict fed (effect of food intake)	1	.0001
	Ad libitum vs restrict fed (strep only)	1	.0002
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
Jejunum %	Ad libitum vs restrict fed (effect of food intake)	1	NS
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
Ileum Weight	Ad libitum vs restrict fed (effect of food intake)	1	.0001
	Ad libitum vs restrict fed (strep only)	1	.01
	Cellulose vs rhubarb (diet)	1	.06
	Autoimmune vs streptozoticin diabetes	1	.00010
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.02
Ileum % Mucosa	Ad libitum vs restrict fed (effect of food intake)	1	NS
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
Colon Weight	Ad libitum vs restrict fed (effect of food intake)	1	.0003
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS

TABLE 4.4 Effect of Dietary Fiber on Blood Parameters

	Cellulose Fiber Diet			Rhubarb Fiber Diet		
	BBd	**Induced Diabetic**	**Restrict-Fed**	**BBd**	**Induced Diabetic**	**Restrict-Fed**
Insulin	0.21 ± 0.05	0.48 ± 0.06	0.47 ± 0.06	0.27 ± 0.1	0.55 ± 0.05	0.67 ± 0.11
C-Peptide	53.4	387.0 ± 55.3	583.0 ± 120.7	157.0	430.0 ± 55.8	692.5 ± 114.2
Glucagon	137.2 ± 7.2	105.9 ± 7.7	103.4 ± 9.4	145.4 ± 12.5	102.9 ± 6.5	106.0 ± 7.5
Fasting Glucose	439.1 ± 44.5	306.7 ± 76.6	147.0 ± 17.5	421.2 ± 43.8	282.6 ± 59.9	147.2 ± 19.5
30 min Glucose	566.8 ± 37.2	424.1 ± 50.2	325.0 ± 21.9	600.6 ± 13.3	414.2 ± 34.1	307.2 ± 24.3
GLP-1 (7-36)amide	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05	1.11 ± 0.04	1.03 ± 0.05	1.07 ± 0.04

Values are mean ± SEM (n=10 rats / treatment). Values for BBd c-peptide do not contain SEM because plasma levels were below the detection limit of the RIA kit used. Values are therefore an n=1 for BBd c-peptide. Statistical differences are described in the chart of orthogonal contrasts.

Orthogonal Contrasts for Blood Parameters (Table 4.4)

Parameter	Source	df	P
Fasting Glucose	Ad libitum vs restrict fed (effect of food intake)	1	.0001
	Ad libitum vs restrict fed (strep only)	1	.002
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
30 Minute	Ad libitum vs restrict fed (effect of food intake)	1	.0001
Glucose	Ad libitum vs restrict fed (strep only)	1	.002
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
Insulin	Ad libitum vs restrict fed (effect of food intake)	1	.001
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	.05
	Autoimmune vs streptozoticin diabetes	1	.001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
C-Peptide	Ad libitum vs restrict fed (effect of food intake)	1	.003
	Ad libitum vs restrict fed (strep only)	1	.01
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.04
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS
Glucagon	Ad libitum vs restrict fed (effect of food intake)	1	.04
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS

Orthogonal Contrasts for Blood Parameters Cont'd

GLP-1(7-36)	Ad libitum vs restrict fed (effect of food intake)	1	NS
	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	.05
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	NS

TABLE 4.5 Effect of Dietary Fiber on L-Glucose and D-Fructose Uptake in Diabetic Rats

	Cellulose Fiber Diet			Rhubarb Fiber Diet			
	BBd	**Induced Diabetic**	**Restrict-Fed**	**BBd**	**Induced Diabetic**	**Restrict-Fed**	**Diet**
L-Glucose							
Jejunum							
16 mM	142.3 ± 14.7	174.0 ± 18.4	222.3 ± 20.4	189.1 ± 24.2	158.7 ± 19.4	203.5 ± 21.1	NS
Ileum							
16 mM	192.1 ± 30.5^{a}	193.6 ± 27.5^{a}	347.7 ± 32.3^{b,y}	209.4 ± 31.0^{a}	175.8 ± 17.7^{a}	265.7 ± 19.2^{b,x}	p<0.05
Fructose							
Jejunum	11.69 ± 0.85	11.69 ± 0.77	13.07 ± 0.57^{y}	11.77 ± 0.69^{a}	13.06 ± 0.36^{a}	15.55 ± 0.36^{b,x}	p<0.05
Ileum	16.53 ± 0.28^{a}	15.00 ± 0.42^{b}	15.39 ± 0.82^{b,y}	13.65 ± 2.48	13.13 ± 0.30	12.20 ± 0.33^{x}	p<0.05

Values are mean ± SEM (n=6 rats / treatment). Units are nmol/100 mg tissue/minute. Superscripts 'x' and 'y' represent the significant difference (p<0.05) between rhubarb and cellulose diets. Superscripts 'a' and 'b' represent a significant difference (p<0.05) between disease state. Comparisons are between BBd and streptozotocin-diabetic ad libitum fed animals and between streptozotocin-diabetic ad libitum and restrict fed animals.

TABLE 4.6 Effect of Dietary Fiber on D-Glucose Uptake Kinetics in Diabetic Rats (corrected for passive permeability and unstirred water layer resistance)

	Cellulose Fiber Diet			Rhubarb Fiber Diet			
	BBd	Induced Diabetic	Restrict-Fed	BBd	Induced Diabetic	Restrict-Fed	Diet
Jejunum							
Vmax	295 ± 37^{a}	810 ± 310^{b}	952 ± 145	384 ± 49^{a}	693 ± 50^{b}	377 ± 236	NS
Km	$0.08 \pm 0.09^{a,x}$	26.8 ± 16.9^{b}	9.1 ± 3.5	1.0 ± 0.3^{y}	3.8 ± 1.0	7.5 ± 8.4	$p<0.05$
Ileum							
Vmax	n/a	458 ± 54^{x}	507 ± 118	756 ± 216	674 ± 76^{y}	611 ± 65	$p<0.05$
Km	n/a	0.3 ± 0.3^{x}	1.3 ± 1.3	16.5 ± 7.1	2.7 ± 1.0^{y}	1.1 ± 0.6	$p<0.05$

Values are mean ± SEM (n=6 rats / treatment). Units for Vmax are nmol/100 mg tissue/minute. Units for Km are mM. n/a, not available (unable to calculate kinetic parameters). NS (non-significant). Superscripts 'x' and 'y' represent the significant difference ($p<0.05$) between rhubarb and cellulose diets.Superscripts 'a' and 'b' represent a significant difference ($p<0.05$) between disease state. Comparisons are between BBd and streptozotocin-diabetic ad libitum fed animals and between streptozotocin-diabetic ad libitum and restrict fed animals.

Orthogonal Contrasts for Proglucagon, SGLT-1 & GLUT2 mRNA (Fig 4.1-Fig 4.4)

Parameter	Source	df	P
Ileal Proglucagon	Ad libitum vs restrict fed (effect of food intake)	1	NS
mRNA	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	.03
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.01
Colonic	Ad libitum vs restrict fed (effect of food intake)	1	NS
Proglucagon	Ad libitum vs restrict fed (strep only)	1	NS
mRNA	Cellulose vs rhubarb (diet)	1	.04
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.05
Jejunal SGLT-1	Ad libitum vs restrict fed (effect of food intake)	1	.0001
mRNA	Ad libitum vs restrict fed (strep only)	1	.0002
	Cellulose vs rhubarb (diet)	1	.007
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.02
Jejunal GLUT2	Ad libitum vs restrict fed (effect of food intake)	1	.02
mRNA	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.07

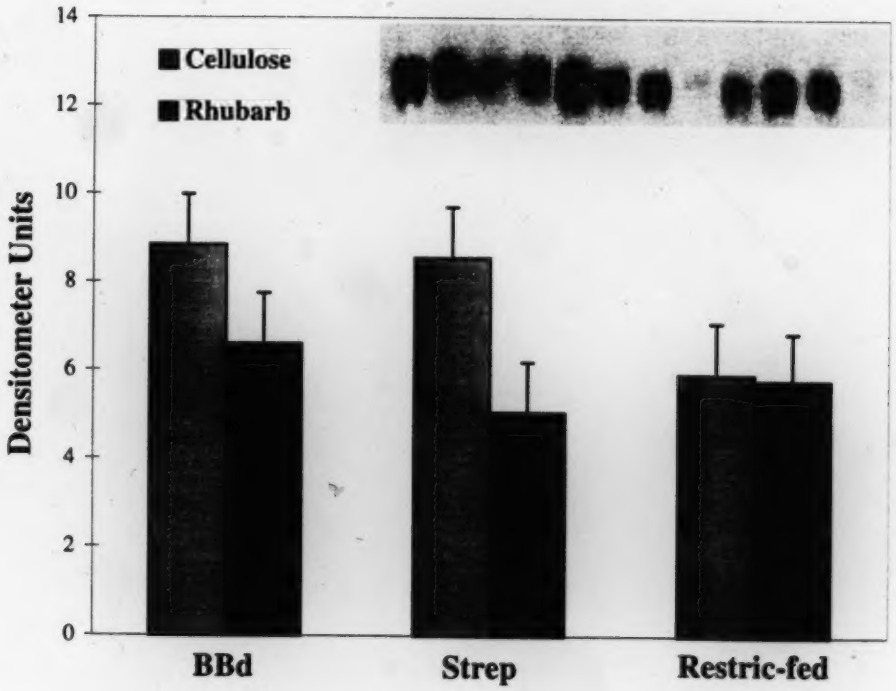

Figure 4.1 Effect of rhubarb and cellulose fiber on ileal proglucagon mRNA expression in diabetic rats. Values are mean ± SEM (n=8 rats / treatment). Statistics are described in the chart of orthogonal contrasts. Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right in duplicate: BBdp cellulose, BBdp rhubarb, Streptozocin-induced diabetic cellulose, Streptozocin-induced diabetic rhubarb, Pairfed cellulose, Pairfed rhubarb. Each lane represents an individual rat.

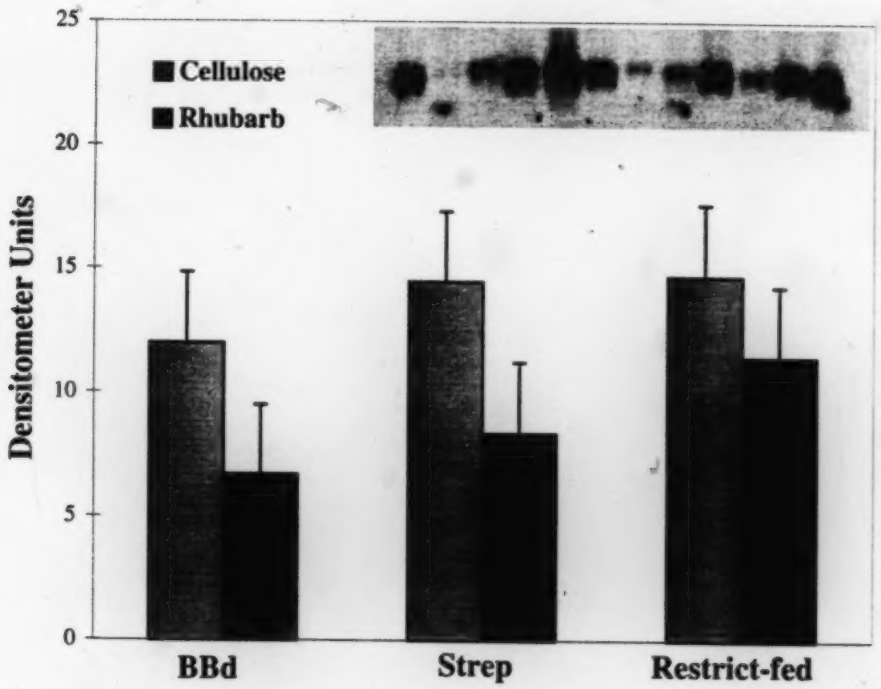

Figure 4.2 Effect of rhubarb and cellulose fiber on colonic proglucagon mRNA expression in diabetic rats. Values are mean ± SEM (n=8 rats / treatment). Statistics are described in the chart of orthogonal contrasts. Inset of autoradiograph. Each lane was loaded with15 ug of total RNA. From left to right in duplicate: BBdp cellulose, BBdp rhubarb, Streptozocin-induced diabetic cellulose, Streptozocin-induced diabetic rhubarb, Pairfed cellulose, Pairfed rhubarb. Each lane represents an individual rat.

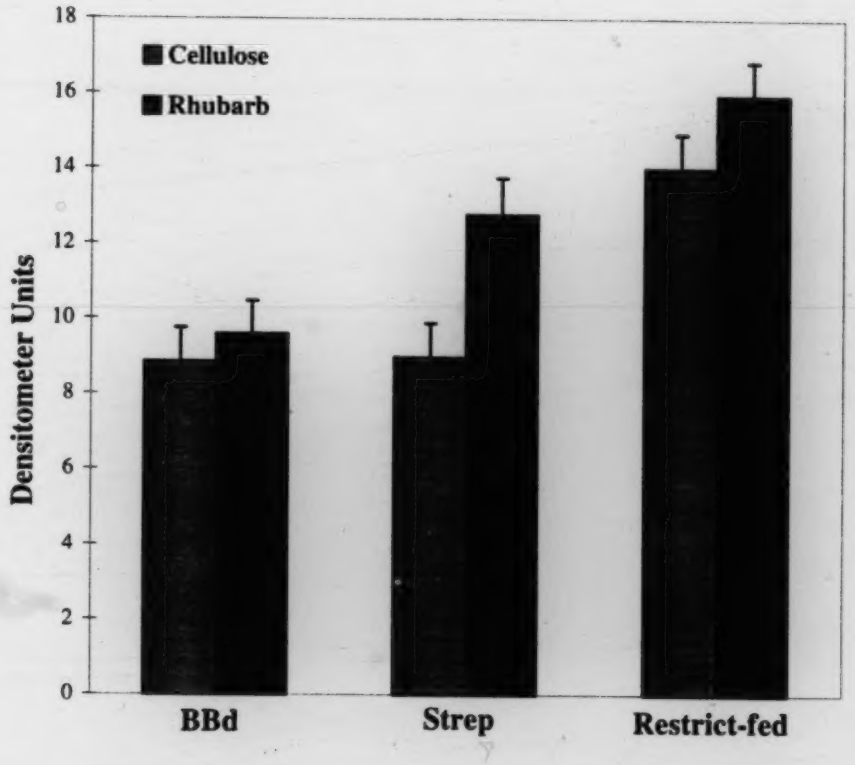

Figure 4.3 Effect of rhubarb and cellulose fiber on jejunal SGLT-1 mRNA expression in diabetic rats. Values are mean ± SEM (n=8 rats / treatment). Each lane was loaded with 15 ug of total RNA. Statistics are described in the chart of orthogonal contrasts.

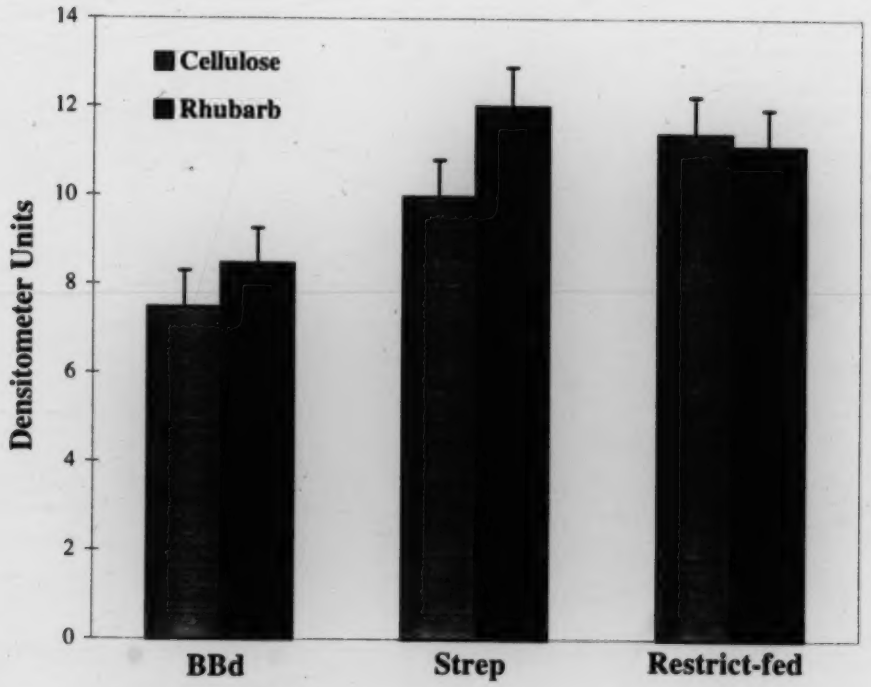

Figure 4.4 Effect of rhubarb and cellulose fiber on jejunal GLUT2 mRNA expression in diabetic rats. Values are mean ± SEM (n=8 rats / treatment). Each lane was loaded with 15 ug of total RNA. Statistics are described in the chart of orthogonal contrasts.

Orthogonal Contrasts for Proglucagon, SGLT-1 and GLUT2 mRNA (Fig. 3 & Fig. 4)

Parameter	Source	df	P
Ileal Proglucagon	Ad libitum vs restrict fed (effect of food intake)	1	NS
mRNA	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	.03
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.01
Colonic	Ad libitum vs restrict fed (effect of food intake)	1	NS
Proglucagon	Ad libitum vs restrict fed (strep only)	1	NS
mRNA	Cellulose vs rhubarb (diet)	1	.04
	Autoimmune vs streptozoticin diabetes	1	NS
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.05
Jejunal SGLT-1	Ad libitum vs restrict fed (effect of food intake)	1	.0001
mRNA	Ad libitum vs restrict fed (strep only)	1	.0002
	Cellulose vs rhubarb (diet)	1	.007
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.02
Jejunal GLUT2	Ad libitum vs restrict fed (effect of food intake)	1	.02
mRNA	Ad libitum vs restrict fed (strep only)	1	NS
	Cellulose vs rhubarb (diet)	1	NS
	Autoimmune vs streptozoticin diabetes	1	.0001
	Cellulose vs rhubarb in ad libitum fed diabetics	1	.07

CHAPTER 5

ONTOGENIC CHANGES IN PROGLUCAGON AND GLUCOSE TRANSPORTER mRNA IN BB DIABETES PRONE AND NORMAL RATS IN RESPONSE TO FEEDING CHOW DIETS[1,2]

INTRODUCTION

The bio-breeding (BB) rat is a useful animal model of human insulin-dependent diabetes mellitus (IDDM) (Parfrey et al, 1989; Yale & Marliss, 1984). The BB rat displays many of the immune irregularities associated with the human disease. As in human IDDM the syndrome is spontaneous and results in the autoimmune destruction of the insulin-producing beta cells of the pancreas (Like et al, 1982; Marliss et al, 1982). It is well established that both forms of diabetes, human and BB are autoimmune.

Investigations into the etiology of IDDM suggest that both genetic and environmental factors are involved (Barnett et al, 1981). It is now well recognized that diet is an important factor influencing development of diabetes in the BB rat (Scott & Marliss, 1991; Butler et al, 1983). Studies suggest that semi-purified diet may be protective whereas laboratory chow may be more diabetogenic in nature (Scott et al, 1988; Hoorfar et al, 1993).

Whether the protective effect of a semi-purified diet is due to the presence of a specific protective component or conversely the absence of a diabetogenic component is yet unknown. It is known, however, that the timing of initial exposure and duration of

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[1]A version of this chapter has been accepted for publication in *Diabetologia*.

[2]This work will be presented at the International Congress of Nutrition, July 1997, Montreal, Quebec and published in part in abstract form [Reimer RA, Field CJ, McBurney MI, Ontogenic changes in proglucagon and glucose transporter mRNA in BB diabetes prone and normal rats in response to feeding chow diets].

exposure influence the protective effect of semi-purified diets. There appears to be a 'critical window' in which exposure to protective diet must occur. In order to decrease the incidence of diabetes, the diet must be introduced prior to 30 days of age and continued for at least 100 days (Issa-Chergui et al, 1988; Scott & Marliss, 1991).

Relatively little is known regarding the factors affecting the development of the intestine in the autoimmune BB rat, particularly surrounding the time of weaning. The fact that the drug, cyclosporin A, often used to delay the onset of autoimmune diabetes in the rat causes a delay in the maturation of the small intestine during weaning in the rat (Cummins et al, 1989) suggests that changes occurring in the intestine play a crucial role in disease incidence. As well, it has been shown that rats weaned to chow diets demonstrate higher rates of growth that those weaned to semi-purified diets (Elliot & Martin, 1984; Hoorfar et al, 1992). Pedersen et al (1994) showed that the risk of developing diabetes was greatest in the animals which displayed the highest body weight at 10-40 days of age. Changes in the absorption of nutrients from the intestine of diabetic prone animals and the subsequent effect on pancreatic function may play a role in the ultimate development of the disease.

Using the diet which produces the highest incidence of diabetes in the BB rat, laboratory chow, we have examined the ontogenic development of the intestine in BBn (normal) and BBdp (diabetes prone) rats at 7, 10, 14, 21, 24 and 30 days of age. Gene expression of the gut hormone, proglucagon, and of intestinal glucose transporters was examined. In addition to characterization of intestinal mass and RNA content, plasma glucose and plasma GLP-1(7-36)amide concentrations were assessed.

MATERIALS AND METHODS

Animals BBdp and BBn rat dams were obtained from the University of Alberta, Dept of Agricultural, Food and Nutritional Science breeding colony. Original breeding pairs were obtained from Health Canada (Animal Resources Division, Health Protection Branch,

Health Canada, Ottawa, ON, Canada). BBdp and BBn rat dams were given free access to standard laboratory chow (Rodent Laboratory Diet PMI #1500, PMI Feeds Inc., St. Louis, MO) and water ad libitum. Litters were culled to 8 pups. Male and female rat pups were removed from the dam and weaned to standard laboratory chow at 21 days of age. Animals were housed in a temperature and humidity controlled room with a 12 hour light/dark cycle. Animals killed at 24 and 30 days of age were group housed from weaning (21 days) until 30 days. Principles of laboratory animal care were followed (NIH publication No. 85-23, revised 1985). The protocol was approved by the University of Alberta Animal Welfare Committee.

Rat pups sacrificed at 7 days and 10 days of age were removed from the dam and asphyxiated with CO_2 and then decapitated. Animals sacrificed from 14d through 30d were anaesthetized and blood taken by cardiac puncture. For all animals the entire small intestine and colon were excised, contents removed and tissues measured and weighed. The small intestine was divided by length into three equal segments. Tissues were immersed in liquid nitrogen and stored at -72 C for later mRNA analysis.

Isolation of Total RNA Total RNA was isolated using Trizol™ (Gibco BRL, Burlington, ON, Canada). Isolation was according to the protocol provided with the reagent. RNA was dissolved in DEPC (diethyl pyrocarbonate) treated water and quantity and purity determined by ultraviolet spectrophotometry at 260 and 280 nm.

Northern Blot Analysis Messenger RNA in all samples was measured using a Northern blot analysis procedure described by Fuller et al (1989) with modifications. Aliquots of 15 µg total RNA were dissolved in 10 uL gel loading buffer (50% deionized formamide (vol/vol), 2M formaldehyde, 1.3% glycerol (vol/vol), 0.02 M morpholinopropanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA and 0.1% bromophenol blue (wt/vol)), boiled for 2 min to denature the RNA and then placed on ice for 5 min. Samples were centrifuged briefly and loaded onto 1% agarose (wt/vol)

gels containing (0.66M) formaldehyde. RNA was then fractionated according to size by electrophoresis in the presence of a recirculating running buffer containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA (5h at 100V). After electrophoresis, the gels were soaked in two changes of 10X standard saline citrate (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and then blotted onto a MSI Nitropure nitrocellulose membrane (MSI Laboratories, Westboro, MA), employing the capillary method described by Southern (1975). The RNA was then fixed onto membranes by baking in vacuo at 80 C for 2h.

Prior to hybridization with the [^{32}P] ATP-labelled cDNA probe, membranes were prehybridized for 2h at 65 C in prehybridization buffer [6X SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS (wt/vol), 5X Denhart's solution (0.5 g Ficoll 400, 0.5 g PVP, 0.5 g BSA (frakcia v)]. Following prehybridization, membranes were incubated for 16h at 65 C in an identical volume of fresh hybridization buffer with the addition of labelled cDNA probe. The cDNA proglucagon probe (donated by Dr. PJ Fuller, Prince Henry's Institute of Medical Research, Melbourne, Australia), GLUT2 cDNA probe (donated by Dr. GI Bell, Howard Hughes Medical Institute, University of Chicago, IL), GLUT5 cDNA probe ((donated by Dr. GI Bell, Howard Hughes Medical Institute, University of Chicago, IL) and SGLT-1 cDNA probe (donated by Dr. N. Davidson, University of Chicago, IL) were labelled by nick translation (Random Primers DNA Labelling System, Life Technologies, Burlington, ON) with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada, Oakville, ON).

Following hybridization, membranes were washed 3 times for 20 min each at room temperature with 2X SSPE, 0.1% (wt/vol) SDS. They were then washed once at 65 C for 20 min with 0.1X SSC, 0.1% (wt/vol) SDS. Membranes were heat sealed in plastic bags and then exposed at -70 C to KODAK XAR5 film (Eastman Kodak, Rochester, NY) using an intensifying screen (Dupont Canada, Mississuaga, ON). For statistical analysis, the signals were quantified using laser densitometry (Model GS-670 Imaging

Densitometer, BioRad Laboratories (Canada) Ltd., Mississuaga, ONT). The 28S and 18S ribosomal bands were quantified from negatives of photographs of the membranes. These bands confirm the integrity of the RNA and were used as a denominator for densitometer values to compensate for any loading discrepancies.

Radioimmunoassay Blood was collected from each rat into a chilled syringe. Aprotinin (Trasylol®, 10,000 K.I.U./ml, Bayer AG, Germany) and EDTA were added to the blood at 500 K.I.U./ml and 1 mg/ml respectively. Blood was centrifuged at 1,600 x g for 15 minutes at 4 C and plasma stored at -70 C until analysis.

GLP-1(7-36)amide GLP immunoreactive peptides were extracted from 2.2 ml of plasma using a SEP-COLUMN containing 200 mg of C_{18} (Cat # RIK-SEPCOL 1, Peninsula Laboratories, Belmont, CA) with Buffer A (0.1% trifluoroacetic acid (Cat # RIK-BA-1, Peninsula Laboratories, Belmont, CA)) and Buffer B (60% acetonitrile (Cat # RIK-BB-1, Peninsula Laboratories, Belmont, CA)) as elution solvents. The extraction was performed according to the protocol provided by Peninsula Laboratories. Radioimmunoassay was performed using a dextran coated charcoal method for separation of bound and free label. The GLP-1(7-36)amide antibody was a gift from Dr. CHS McIntosh of the University of British Columbia. GLP-1(7-36)amide standard and ^{125}I-GLP-1(7-36)amide were purchased from Peninsula Laboratories. The antiserum was c-terminally directed and detected amidated forms of GLP-1 with no significant cross reactivity with non-amidated forms of GLP-1. The intra-assay coefficient of variation was 4.1%.

Plasma Glucose Determinations Plasma glucose was determined using Sigma Diagnostics Glucose (Trinder) Reagent for the enzymatic determination of glucose at 505 nm (Sigma Chemical, St. Louis, MO).

Statistical Analysis All data are expressed as mean ± SEM. Differences between treatments were determined using the two-way ANOVA model in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Statistical significance is defined as $p \leq 0.05$. For ease of presentation, use of the word 'disease' refers to the difference in 'susceptibility to disease' between the BBn and BBdp animals.

RESULTS

Growth Parameters

The effect of disease and age on growth and intestinal weight change are shown in **Table 5.1**. Body weight, total small intestine weight, small intestine weight as a proportion of body weight, colon weight and colon weight as a proportion of body weight increased with increasing age ($p<0.0001$). Only at 21 d and 24 d did BBdp animals weigh less than BBn animals ($p<0.0001$). Two critical time points (21 and 30 days) were associated with higher small intestine weight per body weight in BBdp than in BBn animals. At 30 d BBdp had greater colon weight than BBn ($p<0.0001$). At all ages except 24 d BBdp had higher colon weight per body weight than BBn ($p<0.05$).

Intestinal RNA Content

Concentration (mg/g) of RNA in jejunum, ileum and colon increased significantly with age ($p<0.0001$) but did not differ between BBdp and BBn animals (data not shown). Total RNA from the segments, jejunum, ileum and colon as well as total intestinal RNA increased significantly ($p<0.0001$) with age (**Table 5.2**). 14 d appears to be the critical time point at which BBdp have higher total jejunal and ileal RNA content than BBn ($p<0.0001$). 21 d appears to be the critical time point at which BBdp have higher total colonic and intestinal RNA content than BBn (minimum $p<0.05$).

Blood Parameters

At 30d of age BBdp had significantly ($p<0.0005$) higher plasma GLP-1(7-36)amide than BBn animals (**Figure 5.1**). The increase in GLP-1 was more than two-fold in magnitude.

No significant difference between diseases was found in plasma glucose levels **(Figure 5.2)**. Within BBdp animals 30 d plasma glucose was higher than 14 d values ($p<0.05$). Within BBn animals 30 d plasma glucose was higher than 21 d values ($p<0.05$).

Proglucagon mRNA

At 14 d a significant decrease occurred in the abundance of proglucagon mRNA in the ileum of BBdp and BBn animals ($p<0.0001$) **(Figure 5.3)**. Nonsignificantly at 7 d and significantly at 10d of age BBdp had higher ileal proglucagon mRNA ($p<0.01$) than BBn. By 14 d, with the initial introduction of food this pattern was reversed with BBn now demonstrating higher proglucagon mRNA than BBdp. Accounting for the total potential message in the ileum by multiplying proglucagon mRNA by total ileal RNA content shows that total potential proglucagon mRNA was elevated in 24 d and 30 d BBdp compared to younger BBdp animals ($p<0.0001$) **(Figure 5.4)**. In BBn animals, 21 d was the age at which higher total potential proglucagon mRNA was seen compared to younger animals ($p<0.001$). BBdp animals had lower total potential ileal proglucagon mRNA than BBn at 21d, 24d and 30d ($p<0.05$).

In the colon 14 d again appears to be the critical period at which colonic proglucagon mRNA decreases from earlier ages ($p<0.0001$) **(Figure 5.5)**. BBdp consistently had lower levels of colonic proglucagon mRNA than BBn with this difference reaching significance at 7d and 30d ($p<0.05$). Accounting for the total potential message in the colon by multiplying proglucagon mRNA by total colonic RNA content shows that total potential proglucagon mRNA was significantly increased at 21 d and remained higher through to 30 d in both BBdp and BBn animals ($p<0.001$) **(Figure 5.6).** Within BBdp animals, proglucagon mRNA is higher at 24 d and 30 d ($p<0.0001$) compared to BBn animals. This critical period appears to begin already at 21 d ($p=0.09$).

Glucose Transporter mRNA

Effects of age and disease on glucose transporter mRNA are shown in **Table 5.3**. In

BBdp rats SGLT-1 mRNA was lowest at 7d and peaked at 21d ($p<0.05$). In the BBn rats the increase occurred later with significantly higher levels of mRNA seen at 24d and 30d ($p<0.05$). Disease was significantly different with BBdp animals having higher levels at 21d ($p<0.01$) and BBn showing highest levels at 30d ($p<0.0003$).

In BBdp rats levels of GLUT2 were higher at 10d, 14d and 21d compared to other ages ($p<0.05$). In BBn only 10d were higher than other ages ($p<0.002$). For GLUT2 mRNA 14 d and 21 d again appear to be the critical period at which BBdp have significantly higher GLUT2 mRNA than BBn ($p<0.02$).

In BBdp rats peak levels of GLUT5 were seen at 21d ($p<0.0002$). In BBn rats, levels of GLUT5 reached maximum values at 30d ($p<0.0001$) with 24d also significantly ($p<0.003$) higher than earlier time points. In BBdp animals significantly higher levels of GLUT5 mRNA are seen at 21 d ($p<0.0001$) while BBn animals only exhibit higher levels later on at 24d and 30d ($p<0.01$).

Values obtained for the purpose of accounting for the total potential message for glucose transporter mRNA by multiplying transporter mRNA by total jejunal RNA content are shown in **Table 5.4**. SGLT-1 in both BBdp and BBn was significantly elevated from 21d through to 30d compared to younger ages ($p<0.0001$). At 21d ($p<0.007$) and 24d ($p<0.0001$) BBdp had higher levels of total SGLT-1 than BBn. By 30 d this pattern is reversed with BBn having higher levels of potential SGLT-1 mRNA than BBdp ($p<0.009$).

For total potential GLUT2 mRNA BBdp animals have the highest abundance beginning at weaning (21 d) and remaining elevated through to 30d ($p<0.001$). In BBn the increase was present later on at 24d ($p<0.02$) and again remained elevated at 30d ($p<0.0001$). The significantly higher abundance of potential GLUT2 message in BBdp at 21 d ($p<0.03$) and 24d ($p<0.002$) than BBn again reinforces the changes occurring in the BBdp animals

at the critical period of weaning.

Age patterns for total potential GLUT5 mRNA mimicked those seen with GLUT2. BBdp had higher levels of total GLUT5 at 21d ($p<0.003$) than BBn but as with other transporters, BBn had higher abundance than BBdp at 30d ($p<0.0001$).

DISCUSSION

Dramatic changes in intestinal morphology and enzymology occur from birth to the early post-weaning period. Diet as well as humoral growth factors influence the ontogeny of small intestinal morphology and enzymology (Sagor et al, 1983; Henning, 1985). Changes occurring in the intestine of the autoimmune diabetes prone BB rat in the early post-natal period is of particular interest. Replacing chow diets with semi-purified diets reduces the incidence of IDDM in BB rats provided the diet is introduced at weaning (21 days). This study examined the ontogenic development of the intestinal hormone, proglucagon and intestinal glucose transporters. For the first time we describe dramatic differences in intestinal growth and the expression of proglucagon, SGLT-1, GLUT2 and GLUT5 mRNA in BBn and BBdp rats from 7 to 30 days of age.

Weaning to chow diets produces the highest incidence of diabetes in BB rats compared to feeding a semi-purified diet (Scott et al, 1988; Hoorfar et al, 1993). BBdp rats at 21 and 30 days of age had significantly greater small intestine weight per body weight than BBn animals at these time points. At all ages examined except 24 days of age, colon weight per body weight was significantly higher in BBdp versus BBn animals. These findings suggest that on a body weight basis, BBdp animals have a larger gut and greater capacity for nutrient absorption.

Chow diets may increase gut signalling which stimulates pancreatic β-cell insulin secretion. GLP-1(7-36)amide is a potent insulin secretagogue produced in the distal

ileum and colon (Holst, 1994). Previous work has demonstrated that feeding a high fiber diet upregulates proglucagon mRNA and increases GLP-1 and insulin secretion (Reimer & McBurney, 1996). Colonic proglucagon mRNA adjusted for total colonic RNA content is significantly higher in BBdp rats compared to BBn rats at 24 and 30 days of age. This increase begins, although not significantly, at weaning (21 days). The higher colonic proglucagon mRNA corresponds to the significantly elevated plasma GLP-1(7-36)amide seen in 30 day old BBdp versus BBn animals.

The increased proglucagon mRNA seen at 24 and 30 days of age and beginning to rise already at 21 days in the BBdp rats suggests an increase in plasma levels of GLP-2 as well as GLP-1. Recent studies have demonstrated that prohormone convertases are responsible for the processing of proglucagon to the intestinal peptides, glicentin, oxyntomodulin, GLP-1(1-37/36NH_2), GLP-1(7-37/36NH_2) and GLP-2 (Dhanvantari et al, 1996; Rouille et al, 1995). Therefore an increase in plasma GLP-1(7-36)amide as demonstrated in this study would suggest an increase in plasma GLP-2 as well.

GLP-1 is a potent insulin secretagogue and increased levels in plasma would signal increased insulin secretion from the pancreatic beta cells. GLP-2 has recently been shown to increase D-glucose maximal transport rate in the jejunum (Cheeseman & Tsang, 1996) and stimulate intestinal epithelial proliferation (Drucker et al, 1996). An increase of this nature along with the demonstrated upregulation of glucose transporter mRNA in BBdp animals at 21 days of age would further challenge the secretory function of the pancreatic beta cell. It is well established that increasing insulin secretion by administration of cyclophosphamide accelerates the progression of beta cell destruction (Mordes et al, 1987; Kurasawa et al, 1993). Conversely, by administering exogenous insulin in BBdp rats, the incidence of diabetes can be reduced (Mordes et al, 1987). Transplantation of normal histocompatible islets from Wistar-Furth rats still stimulates the immune attack in BB rats and suggests an antigen normally expressed in pancreatic islets mediates the autoaggressive T-cell attack (Rossini et al, 1985; Ihm et al, 1991). The

increase in proglucagon mRNA, therefore, resulting in increased GLP-1 and GLP-2 production may play an important role in increasing the secretory potential of pancreatic islets and ultimate disease pathogenesis.

In streptozocin induced diabetic models, studies have demonstrated that adaptation of intestinal glucose transport occurs in rats during experimental diabetes (Fedorak et al, 1991; Fedorak et al, 1991). Fedorak et al (1991) demonstrated that in the jejunum adaptation occurs in chronically diabetic rats via an increase in the density of glucose carriers in the upper villus region. The ileum is responsive in both acute and chronic states and results in an increase in carriers in the upper villus region as well as recruitment of carriers in the mid to lower villus region. The authors did not examine whether the increase in functional glucose uptake was associated with an increase in the expression of intestinal glucose transporter mRNA. In the present study we show that as early as weaning, when there are no clinical symptoms of diabetes in the BBdp rat, the expression of SGLT-1, GLUT2 and GLUT5 mRNA is elevated above that observed in normal animals. The increased abundance of these genes increase the potential for the production of glucose transporter protein and increased nutrient uptake.

If increased levels of proglucagon mRNA result in higher levels of GLP-1 and consequently increase pancreatic insulin secretion, the increased expression of intestinal glucose transporters may further exacerbate this phenomenon. Weaning appears to be a "critical period" for the protective or deleterious effects of diet on diabetes incidence in the BB rat. Adjusting for the total RNA content of the intestine further emphasizes the increase in glucose transporter expression seen at 21 days of age in BBdp animals. The higher abundance of transporter mRNA indicates a potential for increased nutrient uptake from the intestine at this "critical" time, suggested by this study to be from 14 days of age, at which time the very first ingestion of the dams diet occurs through to 21 days of age when weaning occurs. In human infants, many retrospective studies have shown a negative correlation between breast feeding as an infant and IDDM occurrence in

adolescence (Mayer et al, 1988; Kostraba et al, 1992). Breast feeding may confer protection by decreasing exposure to foreign antigens in solid food or formula. Indeed, Kostraba et al (1993) showed that individuals with IDDM were exposed to solid foods earlier than non-diabetic individuals.

Several animal studies have shown that higher growth rates are typically seen with chow diets (Elliot & Martin, 1984; Hoorfar et al, 1992). Pedersen et al (1994) recently demonstrated that the risk of developing diabetes later in life is strongly associated with higher body weight at 10-40 days of age. Lower plasma and pancreatic insulin levels were seen in animals that did not develop diabetes (Pedersen et al, 1994). Again this observation has been recognized in human infants. Those infants fed formula grow more rapidly in the first year of life than those who are breast fed and this difference remains after the introduction of solid foods (Heinig et al, 1993; Dewey et al, 1992; Roche et al, 1993).

In summary, we have demonstrated that chow diets dramatically alter normal intestinal development in BBdp versus BBn animals. BBdp animals have significantly greater intestine and colon weight per body weight. These differences are especially evident in the early weaning period from 21 to 30 days of age. Expression of colonic proglucagon mRNA and the secretion of GLP-1(7-36)amide, a potent insulin secretagogue in increased in BBdp animals. At weaning (21 days) glucose transporter mRNA is elevated in BBdp versus BBn animals. Taken together, this evidence suggests that a potentially larger more active gut is associated with increased nutrient (glucose) uptake requiring greater pancreatic involvement. These differences may ultimately contribute to disease pathogenesis.

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Table 5.1 Effect of Disease and Age on Body and Intestinal Weights

Parameter	Group	7d	10d	14d	21d	24d	30d
Body Wt (g)	BBdp	12.9 ± 1.8^{a}	24.2 ± 1.9^{b}	23.3 ± 1.6^{b}	$37.6 \pm 1.6^{c,x}$	$49.1 \pm 1.9^{d,x}$	92.5 ± 1.6^{e}
	BBn	16.1 ± 2.0^{a}	23.4 ± 1.7^{b}	22.5 ± 1.4^{b}	$44.2 \pm 1.7^{c,y}$	$65.2 \pm 1.8^{d,y}$	88.6 ± 1.8^{e}
Total Sm Intestine Wt (g)	BBdp	0.46 ± 0.1^{a}	0.79 ± 0.1^{b}	0.76 ± 0.1^{b}	$1.83 \pm 0.1^{c,x}$	$2.27 \pm 0.1^{d,x}$	$4.40 \pm 0.1^{e,x}$
	BBn	0.57 ± 0.1^{a}	0.69 ± 0.1^{a}	0.70 ± 0.1^{a}	$1.39 \pm 0.1^{b,y}$	$3.40 \pm 0.1^{c,y}$	$3.54 \pm 0.1^{c,y}$
Small Intestine Wt/BW	BBdp	3.6 ± 0.2^{a}	3.4 ± 0.2^{a}	3.3 ± 0.2^{a}	$4.8 \pm 0.2^{b,x}$	4.8 ± 0.2^{b}	$4.8 \pm 0.2^{b,x}$
	BBn	3.7 ± 0.2^{a}	3.1 ± 0.2^{b}	3.1 ± 0.2^{b}	$3.2 \pm 0.2^{b,y}$	5.2 ± 0.2^{c}	$4.0 \pm 0.2^{d,y}$
Colon Wt (g)	BBdp	0.05 ± 0.03^{a}	0.10 ± 0.03^{a}	0.11 ± 0.03^{a}	0.26 ± 0.03^{b}	0.37 ± 0.03^{c}	$0.90 \pm 0.03^{d,x}$
	BBn	0.05 ± 0.03^{a}	0.08 ± 0.03^{a}	0.09 ± 0.02^{a}	0.22 ± 0.03^{b}	0.43 ± 0.03^{c}	$0.60 \pm 0.03^{d,y}$
Colon Wt/BW	BBdp	$0.39 \pm 0.03^{a,x}$	$0.44 \pm 0.03^{ab,x}$	$0.47 \pm 0.03^{b,x}$	$0.68 \pm 0.03^{c,x}$	0.69 ± 0.03^{c}	$0.98 \pm 0.03^{d,x}$
	BBn	$0.30 \pm 0.03^{a,y}$	$0.36 \pm 0.03^{ab,y}$	$0.39 \pm 0.02^{b,y}$	$0.50 \pm 0.03^{c,y}$	0.67 ± 0.03^{d}	$0.67 \pm 0.03^{d,y}$

Values are mean ± SEM (n=8 rats / treatment). Using pdiff values from SAS, superscripts 'a' to 'e' represent significant differences ($p<0.05$) between ages within a row. Using pdiff values from SAS, superscripts 'x' and 'y' represent significant ($p<0.05$) differences between disease groups within an age category.

Table 5.2 Effect of Disease and Age on Intestinal RNA Content

Parameter	Group	7d	10d	14d	21d	24d	30d
Total Jejunal RNA (mg)	BBdp	0.51 ± 0.3^{a}	1.04 ± 0.3^{a}	1.24 ± 0.3^{a}	$3.02 \pm 0.3^{b,x}$	$7.89 \pm 0.3^{c,x}$	$9.41 \pm 0.3^{d,x}$
	BBn	0.68 ± 0.3^{a}	1.09 ± 0.3^{a}	1.01 ± 0.3^{a}	$2.18 \pm 0.3^{b,y}$	$4.14 \pm 0.3^{c,y}$	$7.90 \pm 0.3^{d,y}$
Total Ileal RNA (mg)	BBdp	0.60 ± 0.3^{a}	1.14 ± 0.3^{a}	0.90 ± 0.3^{a}	2.66 ± 0.3^{b}	$6.67 \pm 0.3^{c,x}$	$7.88 \pm 0.3^{d,x}$
	BBn	0.94 ± 0.3^{a}	1.12 ± 0.3^{a}	0.75 ± 0.3^{a}	2.05 ± 0.3^{b}	$4.20 \pm 0.3^{c,y}$	$6.93 \pm 0.3^{d,y}$
Total Colonic RNA (mg)	BBdp	0.17 ± 0.1^{a}	0.27 ± 0.1^{a}	0.32 ± 0.1^{a}	$1.17 \pm 0.1^{b,x}$	$2.56 \pm 0.1^{c,x}$	$3.81 \pm 0.1^{d,x}$
	BBn	0.15 ± 0.1^{a}	0.26 ± 0.1^{a}	0.34 ± 0.1^{a}	$0.78 \pm 0.1^{b,y}$	$1.31 \pm 0.1^{c,y}$	$2.26 \pm 0.1^{d,y}$
Total Intestinal RNA (mg)	BBdp	1.28 ± 0.6^{a}	2.42 ± 0.6^{a}	2.24 ± 0.6^{a}	$6.86 \pm 0.6^{b,x}$	$17.15 \pm 0.6^{c,x}$	$21.10 \pm 0.6^{d,x}$
	BBn	1.75 ± 0.6^{a}	2.44 ± 0.6^{a}	2.10 ± 0.6^{a}	$5.01 \pm 0.6^{b,y}$	$9.62 \pm 0.6^{c,y}$	$17.07 \pm 0.6^{d,y}$

Values are mean ± SEM (n=8 rats / treatment). Using pdiff values from SAS, superscripts 'a' to 'e' represent significant differences ($p<0.05$) between ages within a row. Using pdiff values from SAS, superscripts 'x' and 'y' represent significant differences ($p<0.05$) between disease groups within an age category.

Table 5.3 Effect of Disease and Age on Glucose Transporter mRNA Abundance in 15 µg Total RNA

Parameter	Group	7d	10d	14d	21d	24d	30d
SGLT-1 mRNA	BBdp	1.80 ± 0.12^{a}	1.71 ± 0.12^{a}	1.72 ± 0.13^{a}	$2.19 \pm 0.12^{b,x}$	1.93 ± 0.12^{b}	$1.95 \pm 0.12^{b,x}$
	BBn	1.68 ± 0.13^{a}	1.48 ± 0.12^{a}	1.74 ± 0.12^{a}	$1.65 \pm 0.13^{a,y}$	2.13 ± 0.12^{b}	$2.67 \pm 0.12^{c,y}$
GLUT2 mRNA	BBdp	0.24 ± 0.09^{a}	0.52 ± 0.10^{b}	$0.82 \pm 0.10^{b,x}$	$0.48 \pm 0.08^{b,x}$	0.32 ± 0.08^{ab}	0.27 ± 0.08^{ab}
	BBn	0.22 ± 0.19^{a}	0.65 ± 0.10^{b}	$0.44 \pm 0.10^{c,y}$	$0.26 \pm 0.08^{ac,y}$	0.30 ± 0.09^{ac}	0.39 ± 0.08^{ac}
GLUT5 mRNA	BBdp	1.52 ± 0.18^{a}	1.44 ± 0.20^{a}	1.69 ± 0.18^{a}	$2.23 \pm 0.16^{b,x}$	$1.24 \pm 0.0.16^{a,x}$	$1.02 \pm 0.16^{a,x}$
	BBn	1.07 ± 0.17^{ab}	1.89 ± 0.18^{ac}	1.75 ± 0.18^{abc}	$1.42 \pm 0.18^{b,y}$	$1.97 \pm 0.16^{c,y}$	$2.66 \pm 0.16^{d,y}$

Values are mean ± SEM (n=8 rats / treatment). Using pdiff values from SAS, superscripts 'a' to 'e' represent significant differences ($p<0.05$) between ages within a row. Using pdiff values from SAS, superscripts 'x' and 'y' represent significant differences ($p<0.05$) between disease groups within an age category.

Table 5.4 Effect of Disease and Age on Total Potential Glucose Transporter mRNA Abundance in Total Jejunal RNA

Parameter	Group	7d	10d	14d	21d	24d	30d
SGLT-1 mRNA	BBdp	9.2 ± 7.6^{a}	18.5 ± 7.6^{a}	21.4 ± 8.5^{a}	$67.4 \pm 7.6^{b,x}$	$150.9 \pm 7.6^{c,x}$	$183.0 \pm 7.6^{d,x}$
	BBn	11.7 ± 8.5^{a}	16.6 ± 7.6^{ab}	17.6 ± 7.6^{ab}	$33.9 \pm 8.5^{b,y}$	$98.2 \pm 7.6^{c,y}$	$210.7 \pm 7.6^{d,y}$
GLUT2 mRNA	BBdp	1.3 ± 2.7^{a}	5.7 ± 2.5^{a}	11.5 ± 2.8^{a}	$13.5 \pm 2.5^{b,x}$	$24.7 \pm 2.5^{c,x}$	24.8 ± 2.5^{c}
	BBn	1.6 ± 2.7^{a}	7.3 ± 2.7^{ab}	4.8 ± 2.8^{a}	$6.5 \pm 2.5^{ab,y}$	$11.1 \pm 2.7^{b,y}$	31.6 ± 2.5^{c}
GLUT5 mRNA	BBdp	8.2 ± 12.3^{a}	14.8 ± 13.6^{a}	22.0 ± 12.3^{a}	$67.6 \pm 11.0^{bc,x}$	98.6 ± 11.0^{c}	$95.1 \pm 11.0^{c,x}$
	BBn	7.0 ± 12.0^{a}	21.3 ± 12.3^{a}	18.4 ± 12.3^{a}	$32.0 \pm 12.3^{a,y}$	87.0 ± 11.0^{b}	$208.4 \pm 11.0^{c,y}$

Values are mean ± SEM (n=8 rats / treatment). Using pdiff values from SAS, superscripts 'a' to 'e' represent significant differences ($p<0.05$) between ages within a row. Using pdiff values from SAS, superscripts 'x' and 'y' represent significant differences ($p<0.05$) between disease groups within an age category.

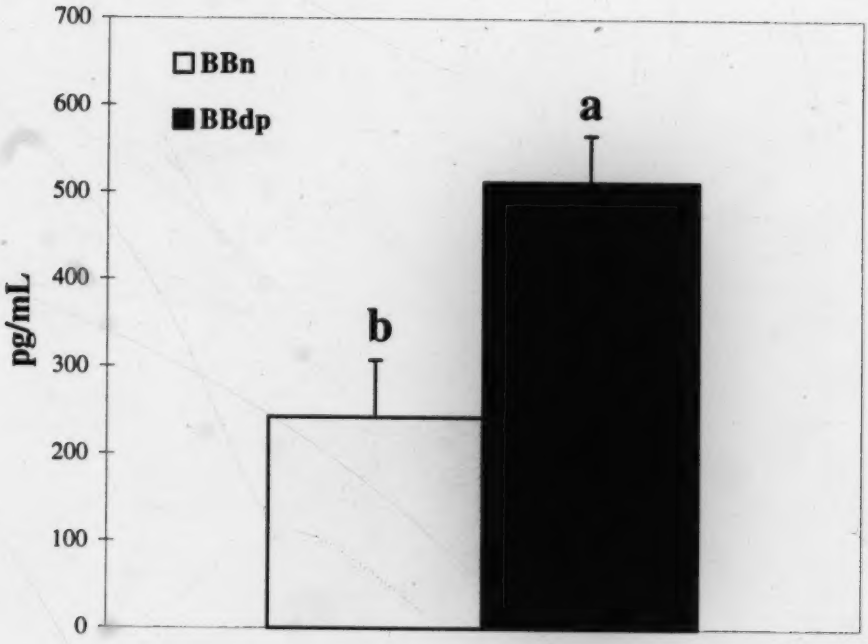

Figure 5.1 Effect of age and disease on non-fasting plasma GLP-1(7-36) amide in 30 day old BB rats. Values are mean ± SEM (n=10 rats / treatment). One-way ANOVA in SAS was used to determine statistical significance. Values with different letters are significantly different ($p<0.0005$).

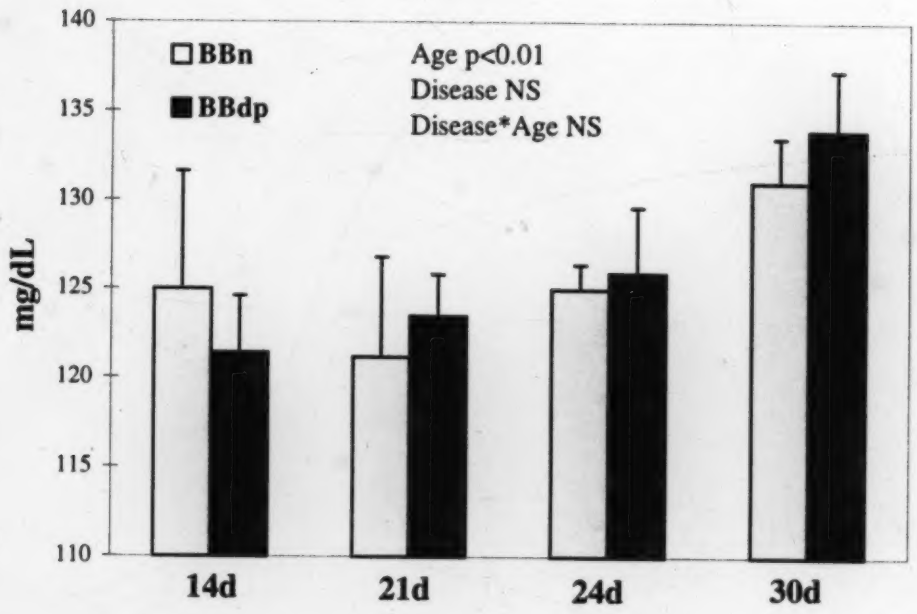

Figure 5.2 Effect of age and disease on non-fasting plasma glucose levels in BB rats. Values are mean ± SEM (n=5-10 rats / treatment). Significant effects are expressed in the figure.

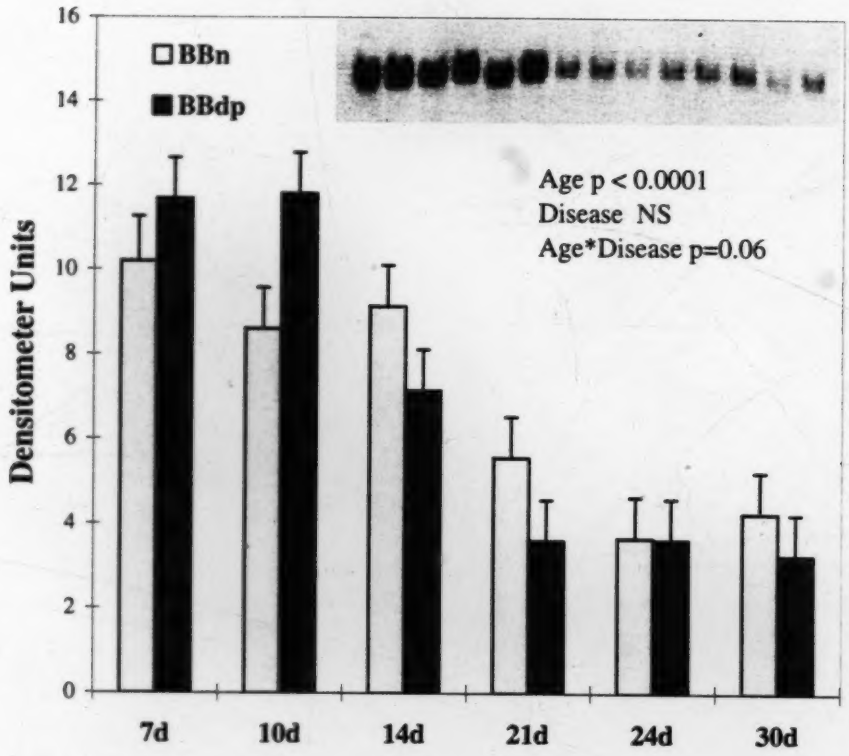

Figure 5.3 Effect of age and disease on ileal proglucagon RNA expression in BB rats. Values are mean ± SEM (n=6 rats / treatment). Significant effects are expressed in the figure. Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right: 7d BBdp, 7d BBdp, 7d BBn, 10d BBdp, 10d BBn, 10d BBn, 14d BBdp, 14d BBn, 21d BBdp, 21d BBn, 24d BBdp, 24d BBn, 24d BBn, 30d BBdp, 30d BBn.

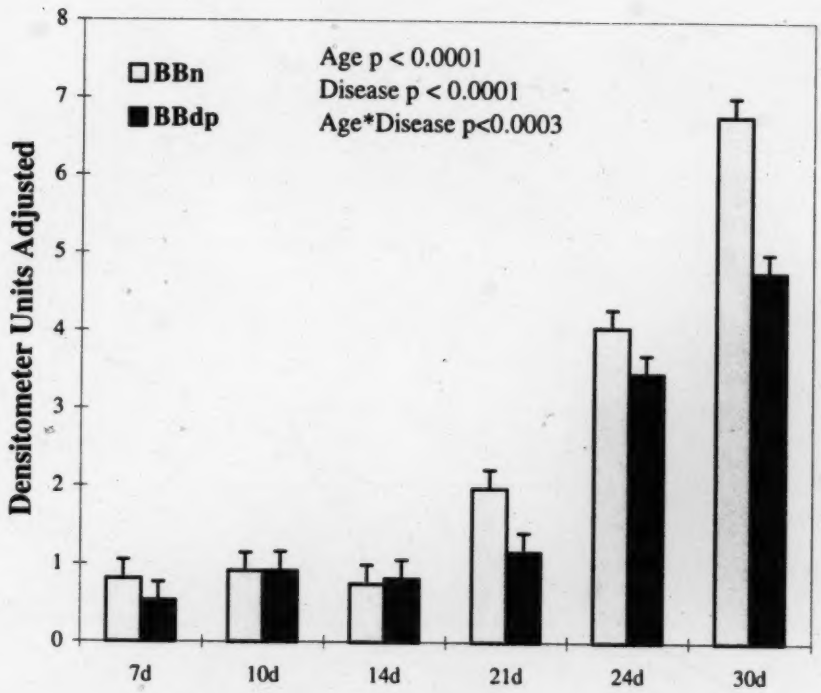

Figure 5.4 Effect of age and disease on total potential ileal proglucagon mRNA abundance in total ileal RNA in BB rats. Values were obtained by multiplying total ileal RNA by ileal proglucagon mRNA levels measured in 15 ug of RNA. Values are mean ± SEM (n=6 rats / treatment). Significant effects are expressed in the figure.

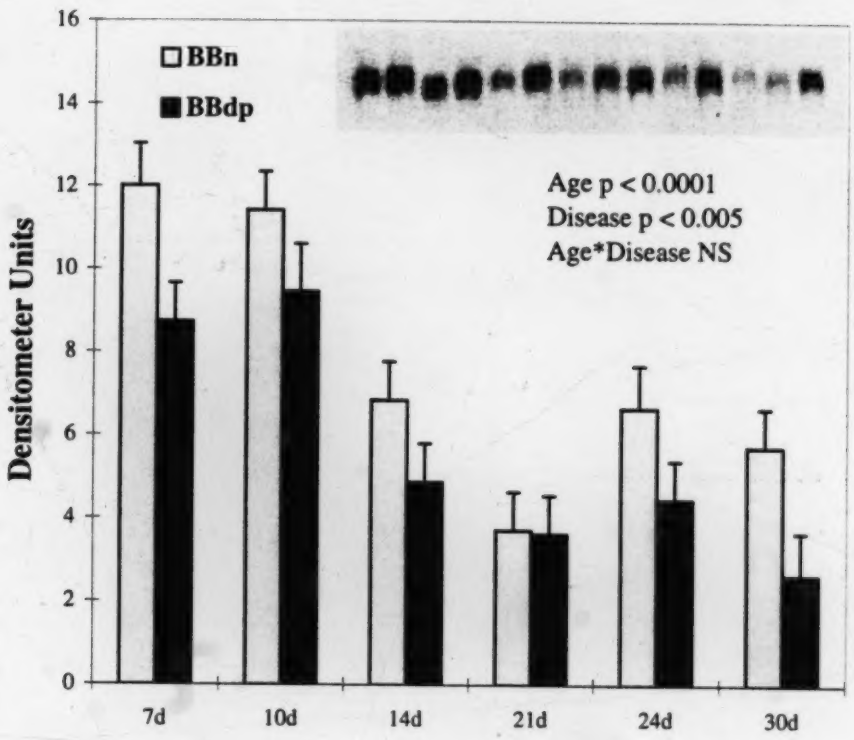

Figure 5.5 Effect of age and disease on colonic proglucagon mRNA expression in BB rats. Values are mean ± SEM (n=6 rats /treatment). Significant effects are expressed in the figure. Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right: 7d BBdp, 7d BBn, 10d BBdp, 10d BBn, 14d BBdp, 14d BBn, 21d BBdp, 21d BBdp, 21d BBn, 24d BBdp, 24d BBn, 30d BBdp, 30d BBdp 30d BBn.

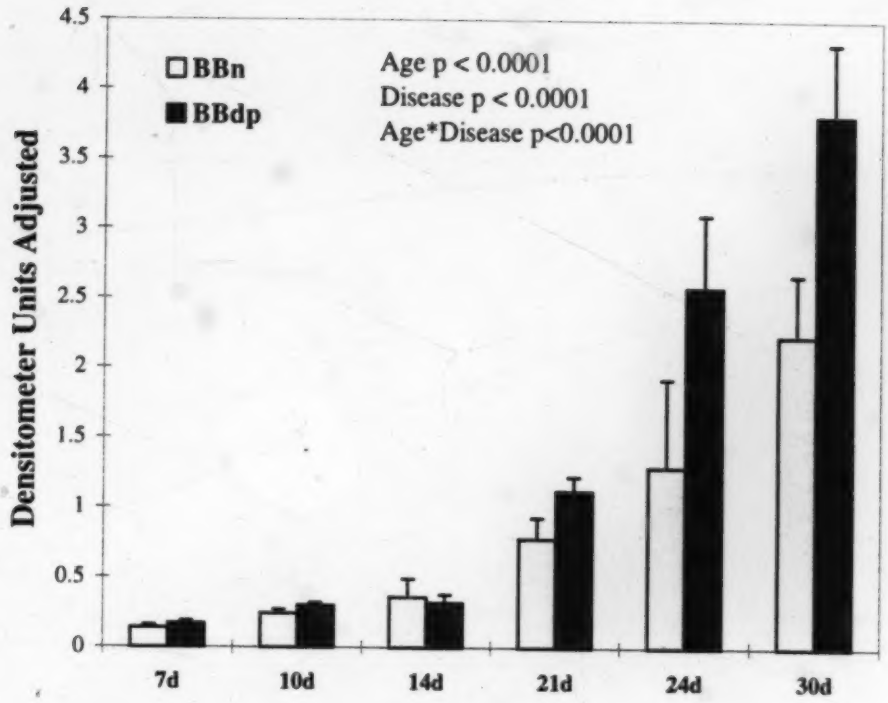

Figure 5.6 Effect of age and disease on total potential colonic proglucagon mRNA abundance in total colonic RNA in BB rats. Values were obtained by multiplyingtotal colonic RNA by colonic proglucagon mRNA levels measured in 15 ug of RNA. Values are mean ± SEM (n=6 rats / treatment). Significant effects are expressed in the figure.

CHAPTER 6

PROGLUCAGON AND GLUCOSE TRANSPORTER mRNA IS ALTERED BY DIET AND DISEASE IN 30 DAY OLD BB DIABETES PRONE AND NORMAL RATS[1]

INTRODUCTION

Early diet and feeding practices have been linked to the etiology of insulin-dependent diabetes mellitus (IDDM) (Kostraba et al, 1993). Retrospective studies assessing early infant nutrition show an increased risk of disease development with early exposure to foods other than breast milk (Mayer et al, 1988; Kostraba et al, 1993). Studies in the BB rat, a valuable model of human IDDM suggest that semi-purified diets may be protective against disease development whereas standard laboratory chow appears to be more diabetogenic in nature (Scott et al, 1988; Hoorfar et al, 1991). The precise mechanism by which semi-purified diets confer protection is yet unknown. A 'critical window' surrounding weaning appears important for disease incidence (Scott & Marliss, 1991) in that diabetes incidence is decreased if the semi-purified diet is introduced prior to 30d and continued for at least 100d (Issa-Chergui et al, 1988; Scott & Marliss, 1991).

The drug, cyclosporin A, has been shown to prevent autoimmune diabetes in animals (Haberstroh et al, 1995; Marco et al, 1996). Cummins et al (1989) demonstrated that cyclosporin A causes a delay in the maturation of the small intestine during weaning in the rat. Delays in morphological and enzymological development decrease nutrient uptake and hormone production (Tsubol et al, 1981; Thomson & Keelan, 1986). Changes in the ontogeny of gut associated immune function and intestinal morphology and function may be involved in disease pathogenesis.

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[1]A version of this chapter has been submitted for publication to *Pediatric Research*.

It has been established that suppressing endogenous insulin secretion can reduce the incidence of diabetes in the BBdp rat (Mordes et al, 1987; Kurasawa et al, 1993). Insulin secretion can be suppressed by slowing glucose transport and/or decreasing the postprandial release of intestinal incretins. L-cells in the distal ileum and colon contain the proglucagon gene which is post-translationally cleaved into several peptides. GLP-1 is a potent insulin secretagogue (Holst, 1994) and delays gastric emptying (Willms et al, 1995). GLP-2 has been shown to increase jejunal glucose transport (Cheeseman & Tsang, 1996). Therefore, changes in proglucagon expression may influence glucose absorption and insulin secretion to ultimately affect the incidence of diabetes.

Cereal-based chow diets contain up to 75% of carbohydrate as complex carbohydrate and contain more fermentable dietary fiber which may stimulate GLP-1 release and postprandial insulin secretion (Reimer & McBurney, 1996). The presence of complex carbohydrate which slows glucose absorption in the small intestine (Wolever, 1991) can also reach the large intestine in significant amounts (Stephen, 1991). The observation that addition of 12% crude protein wheat flour (76% complex carbohydrate) was diabetogenic (Hoorfar et al, 1993) but addition of 10% gluten to semi-purified diets was not suggests that carbohydrate type may be important. McBurney (1991) was able to demonstrate that dietary carbohydrate intake affects the amount of fermentable carbohydrate reaching the large intestine and that different rates and extent of fermentation exist for complex carbohydrate. End products of large bowel fermentation, short chain fatty acids (SCFA), increase intestinal cell proliferation and gastrointestinal digestive development (Sakata, 1989; Koruda et al, 1988). Since wheat products, containing fermentable carbohydrate are encountered at weaning, the role of these substances in diabetogenesis should be further investigated.

In this study we examined the expression of proglucagon and the brush border glucose and fructose transporters, SGLT-1 and GLUT5, respectively, in BBdp and BBn rats at 30d of age fed one of three nutritionally complete diets. Chow produces high incidence,

casein-based SP diet low incidence, and soy-based SP diet an intermediate incidence of diabetes (Scott et al, 1985).

MATERIALS AND METHODS

Animals and Diets Female and male BBdp and BBn rats were obtained from the university of Alberta, Department of Agricultural, Food and Nutritional Science rat colony. Original breeding pairs were obtained from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, ON, Canada). Animals were housed in a temperature and humidity controlled room with a 12hr light/dark cycle. Weanling rats were housed in groups of 2-4 animals per cage. The protocol was approved by the University of Alberta Animal Welfare Committee.

Composition of the experimental diets is given in **Table 6.1**. The non-purified chow diet (NIH-07 Rodent Diet) was purchased from Ziegler Brothers Inc (Gardners, PA, USA). The two semi-purified diets were made in our laboratory with either soy or casein as the protein source.

Dams were fed one of the three experimental diets beginning one week prior to parturition. Pups were weaned from the dams onto the same diet as their respective dams at 21d. Rats were fed until 30 days of age. After an overnight fast animals were killed by cervical dislocation. The entire small intestine and colon were excised, contents removed and tissues measured and weighed. Stomaches were excised and contents removed. The small intestine was divided by length into three equal segments. Approximately 3 cm of distal duodenum, jejunum and ileum and 3 cm of proximal colon were immersed in liquid nitrogen for later mRNA analysis.

Isolation of Total RNA Total RNA was isolated using Trizol™ (Gibco BRL, Burlington, ON, Canada). Isolation was according to the protocol provided with the reagent. RNA was dissolved in DEPC (diethyl pyrocarbonate) treated water and quantity

and purity determined by ultraviolet spectrophotometry at 260 and 280 nm.

Northern Blot Analysis Messenger RNA in all samples was measured using a Northern blot analysis procedure described by Fuller et al (1989) with modifications. Aliquots of 15 ug total RNA were dissolved in 10 uL gel loading buffer (50% deionized formamide (vol/vol), 2M formaldehyde, 1.3% glycerol (vol/vol), 0.02 M morpholinopropanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA and 0.1% bromophenol blue (wt/vol)), boiled for 2 min to denature the RNA and then placed on ice for 5 min. Samples were centrifuged briefly and loaded onto 1% agarose (wt/vol) gels containing (0.66M) formaldehyde. RNA was then fractionated according to size by electrophoresis in the presence of a recirculating running buffer containing 0.02 M MOPS, 5 mM sodium acetate and 1 mM EDTA (5h at 100V). After electrophoresis, the gels were soaked in two changes of 10X standard saline citrate (SSC) (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0) and then blotted onto a MSI Nitropure nitrocellulose membrane (MSI Laboratories, Westboro, MA), employing the capillary method described by Southern (1975). The RNA was then fixed onto membranes by baking in vacuo at 80 C for 2h.

Prior to hybridization with the [^{32}P] ATP-labelled cDNA probe, membranes were prehybridized for 2h at 65 C in prehybridization buffer [6X SSPE (0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS (wt/vol), 5X Denhart's solution (0.5 g Ficoll 400, 0.5 g PVP, 0.5 g BSA (frakcia v)]. Following prehybridization, membranes were incubated for 16h at 65 C in an identical volume of fresh hybridization buffer with the addition of labelled cDNA probe. The cDNA proglucagon probe (donated by Dr. PJ Fuller, Prince Henry's Institute of Medical Research, Melbourne, Australia), GLUT5 cDNA probe (donated by Dr. GI Bell, Howard Hughes Medical Institute, University of Chicago, IL) and SGLT-1 cDNA probe (donated by Dr. N Davidson, University of Chicago, IL) were labelled by nick translation (Random Primers DNA Labelling System, Life Technologies, Burlington, ON) with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada,

Oakville, ON).

Following hybridization, membranes were washed 3 times for 20 min each at room temperature with 2X SSPE, 0.1% (wt/vol) SDS. They were then washed once at 65 C for 20 min with 0.1X SSC, 0.1% (wt/vol) SDS. Membranes were heat sealed in plastic bags and then exposed at -70 C to KODAK XAR5 film (Eastman Kodak, Rochester, NY) using an intensifying screen (Dupont Canada, Mississuaga, ON). For statistical analysis, the signals were quantified using laser densitometry (Model GS-670 Imaging Densitometer, BioRad Laboratories (Canada) Ltd., Mississuaga, ONT). The 28S and 18S ribosomal bands were quantified from negatives of photographs of the membranes. These bands confirm the integrity of the RNA and were used as a denominator for densitometer values to compensate for any loading discrepancies.

Statistical Analysis All data are expressed as mean ± SEM. A significant effect of gender was not found therefore genders were combined and differences between treatments were determined using the two-way ANOVA model (diet and disease) in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Significant differences between groups were determined using pdiff values in SAS. Statistical significance is defined as $p \leq 0.05$.

RESULTS

Growth Parameters

The effects of diet and disease on growth and intestinal characteristics are shown in **Table 6.2**. Diet ($p<0.0001$) and diet*disease ($p<0.0001$) were significant for body weight. BBn animals fed chow had higher body weight than BBdp animals fed chow ($p<0.0001$). BBn fed soy had lower body weight than BBdp fed soy ($p<0.002$). Within BBn, chow fed animals had significantly higher body weight than casein or soy fed animals ($p<0.0001$).

Diet ($p<0.05$) and diet*disease ($p<0.001$) were significant for stomach weight. Only chow fed BBn had higher stomach weight than their BBdp counterparts ($p<0.0003$). Within BBn chow fed animals had significantly greater stomach weight than casein or soy ($p<0.001$).

Only diet*disease was significant for total small intestinal weight ($p<0.01$). Soy BBn had higher intestine weight than soy BBdp ($p<0.003$). Within BBn, chow had significantly lower intestine weight than soy ($p<0.003$).

Expressing intestine weight per body weight resulted in significant diet ($p<0.0004$) and diet*disease ($p<0.0001$) effects. Chow fed BBdp had higher intestine weight per body weight than chow fed BBn ($p<0.0004$). This parameter was lower for BBdp fed soy compared to soy fed BBn ($p<0.0001$). Within BBn chow fed animals had significantly lower intestine weight per body weight compared to casein and soy animals ($p<0.001$).

Small intestine length was significantly affected by diet ($p<0.0007$), disease ($p<0.001$) and diet*disease ($p<0.002$). BBn chow had longer intestines than BBdp chow ($p<0.0001$). Within BBdp diet significantly altered length with chow having the shortest intestine, soy the longest and casein intermediate length ($p<0.05$). Within BBn, casein had shorter intestine length than chow or soy ($p<0.001$).

Diet ($p<0.007$) and disease ($p<0.05$) were significant effects for colon weight. Soy BBdp had greater colon weight than soy BBn ($p<0.009$). Soy BBdp had greater colon weight than casein and chow BBdp ($p<0.006$).

Diet ($p<0.001$) and diet*disease ($p<0.02$) were significant effects for colon weight per body weight. BBdp chow had greater colon weight per body weight than chow BBn ($p<0.005$). Within BBn chow had significantly less than casein or soy ($p<0.001$).

Intestinal RNA Content

Total jejunal RNA was significantly affected by diet ($p<0.004$) and diet*disease ($p<0.01$) **(Figure 6.1)**. Soy BBn had higher total jejunal RNA than soy BBdp ($p<0.004$). Within BBdp chow had the highest total RNA compared to casein and soy ($p<0.004$). Within BBn, soy had significantly higher total jejunal RNA compared to casein ($p<0.003$).

No significant effects were found for total colonic RNA **(Figure 6.2)**.

Proglucagon mRNA

Disease was significant for levels of proglucagon mRNA ($p<0.0001$). BBdp animals fed all three diets had significantly lower levels of proglucagon mRNA ($p<0.01$). Chow fed BBdp animals tended ($p=0.09$) to have higher proglucagon mRNA than soy BBdp **(Figure 6.3)** .

Correcting for total colonic RNA content produced a significant disease effect ($p<0.0001$). BBdp fed casein and soy had significantly lower total proglucagon than BBn fed casein and soy ($p<0.001$). An effect of disease no longer existed between chow BBdp and chow BBn after correction for total RNA. Within BBn casein had higher total proglucagon than chow ($p<0.05$). Within BBdp chow tended ($p=0.09$) to have higher abundance than soy **(Figure 6.4)**.

Glucose Transporter mRNA

Diet, disease and diet*disease (minimum $p<0.002$) were significant effects for SGLT-1 mRNA **(Table 6.3)**. Chow BBn had greater abundance of SGLT-1 mRNA than BBdp chow ($p<0.0001$). Soy BBn had lower abundance of SGLT-1 mRNA than soy BBdp ($p<0.05$). Within BBn chow had the highest abundance of mRNA, soy the lowest and casein intermediate ($p<0.003$).

Diet, disease and diet*disease were significant effects (minimum $p<0.003$) for GLUT5

mRNA (**Table 6.3**). Chow BBn had greater abundance of GLUT5 mRNA than chow BBdp ($p<0.0001$). As for SGLT-1, BBn chow had the highest abundance of GLUT5 mRNA, soy the lowest and casein intermediate ($p<0.005$).

Adjusting SGLT-1 mRNA for total jejunal RNA content produced significant effects for diet ($p<0.0003$) and disease ($p<0.02$) (**Table 6.4**). BBn chow had higher total SGLT-1 message than BBdp chow ($p<0.01$). Chow BBdp had higher total SGLT-1 than casein BBdp ($p<0.05$). Within BBn chow had significantly greater abundance of total SGLT-1 mRNA than casein or soy ($p<0.001$).

Adjusting GLUT5 mRNA for total jejunal RNA content resulted in significant effects of diet, disease and diet*disease (minimum $p<0.01$) (**Table 6.4**). BBn chow had significantly greater GLUT5 than BBdp chow ($p<0.0001$). Within BBn chow had significantly greater total GLUT5 message compared to casein and soy ($p<0.0003$).

DISCUSSION

Growth and maintenance of the small intestine is regulated by numerous factors including dietary nutrients (Tsuoboi et al, 1981; Castillo et al, 1990), luminal secretions (Henning, 1987), systemic hormones (Liu et al, 1992; Yeh & Moog, 1975), and locally produced growth factors (Durant et al, 1996). In the BB rat diet is known to be an important environmental factor governing disease incidence. Our data suggests that weaning diet and genetic predisposition to diabetes affect the mass of the intestine as a proportion of whole body mass.

Soy and casein weaning diets have been shown to confer more protection against diabetes incidence than chow in BBdp rats (Scott et al, 1988; Hoorfar et al, 1991). BBdp animals had larger small intestinal weight and colonic weights per body weight than BBn fed chow diets whereas intestinal masses were similar between BBn and BBdp animals fed

casein and soy diets. Growth of intestinal mass differs between BBn and BBdp animals weaned onto different diets and may have implications for gut associated immune function and nutrient transport.

Intestinal adaptation can occur at the level of the intestine as a whole or at the enterocyte. The increased mass of intestine as a proportion of whole body mass in BBdp rats fed chow is one mechanism by which diabetes may act to nonspecifically increase intestinal glucose absorption. Indeed, Fedorak et al (1989) demonstrated that intestinal glucose transport is increased in diabetes. Changes in SGLT-1 and GLUT5 mRNA suggest that mechanisms specific to the enterocyte may also play a role in increased glucose absorption. Future studies should examine the effect of casein and soy at 21d taking into account that part of the protective effect of these diets may be that the normal peak in glucose transporter mRNA occurs later on, ie. 30d, after the critical weaning period.

Proglucagon is produced in the distal ileum and colon and is the precursor for GLP-1, a potent insulin secretagogue (Holst, 1994). Proglucagon mRNA results in the production of enteroglucagon, thought to be trophic to the gut (Sagor et al, 1983); GLP-1, a potent insulin secretagogue (Mojsov et al, 1987; Holst et al, 1987) and GLP-2, which increases glucose transport in the jejunum (Cheeseman & Tsang, 1996). Since prohormone convertase PC1 is solely responsible for the cleavage of proglucagon into its respective post-translational products (Dhanvanrati et al, 1995), one would expect concomitant increased secretion of all intestinal proglucagon-derived peptides theoretically resulting in increased intestinal transport capacity and a greater stimulus for insulin secretion. Levels of proglucagon mRNA in the colon were significantly lower for BBdp versus BBn animals. Chow tended to increase proglucagon expression versus BBn whereas casein and soy did not. Adjusting for total colonic RNA removed the effect of disease on chow fed animals whereas soy and casein fed BBdp remained significantly lower than their BBn counterparts. The lower proglucagon mRNA found in BBdp animals relative to BBn controls on soy and casein but not chow suggests that BBdp rats fed semi-purified

diets may be subject to less pancreatic challenge early on in life.

In summary we have shown that weaning onto diets varying in diabetogenic potential alters intestinal growth and hormone expression in the BB rat. In normal rats, feeding chow results in greater intestinal growth and abundance of glucose transporter mRNA. BBdp animals fed chow respond differently than those fed casein and soy compared to their respective BBn groups. In fact, the lack of an effect of diet in the BBdp rat on many parameters including SGLT-1, GLUT5 and proglucagon mRNA and the weight of the stomach, small intestine, colon weight per body weight and overall body weight may suggest that the intestine of the BBdp rat is unresponsive to diet. This study has implications not only for the understanding of intestinal adaptation in the diabetes prone rat but also suggests that diet significantly influences the development of the normal rat intestine.

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TABLE 6.1 Composition of the Experimental Diets[1]

Semi-purified (Casein/Soy)[2,3]		NIH-07 (Chow)[4,5]	
Ingredient	**% (wt/wt)**	**Ingredient**	**% (wt/wt)**
Carbohydrate	65	Carbohydrate	59
Fat	5	Fat	5
Protein	20	Protein	23
Fiber (crude)	5	Fiber (crude)	6

[1]Composition as provided by suppliers for lot number used; nonpurified diet, PMI Feeds Inc., St. Louis, MO; and purified diet, ICN, Montreal, Quebec, Canada.

[2]Protein provided as purified high nitrogen casein; carbohydrates (g/kg diet) as sucrose 500 and cornstarch 150.

[3]Supplemented (per kg diet) with 10g AIN-Vitamin mix; 35g AIN-Mineral Mix; 3g DL-methionine and 2g choline bitartrate.

[4]Macronutrients provided from ground yellow corn, soybean meal, dried beet pulp, fish meal, ground oats, brewer's dried yeast, alfalfa meal, cane molasses, wheat germ, dried whey, meat meal, and wheat middlings.

[5]Micronutrient content of the diet (per kg of diet): 10 g calcium; 5.6 g chloride; 4 g sodium; 6 g phosphorus; 11 g potassium; 2 g magnesium 35 mg fluorine; 0.2 g iron; 70 mg zinc; 60 mg manganese; 20 mg copper; 1 mg cobalt; 1 mg iodine; 1 mg chromium; 2 mg selenium; 5295 RE vitamin A; 15 mg thiamin; 8 mg riboflavin; 95 mg niacin; 24 mg pantothenic acid; 0.2 g choline; 6 mg folic acid; 6 mg pyridoxine; 0.07 mg biotin; 0.02 mg vitamin B_{12}; 0.11 mg vitamin D; and 0.2 g vitamin E.

[6]Calculated as the amount remaining after subtracting for protein, fat, fiber and ash.

[7]As alphacel (non-nutritive bulk).

TABLE 6.2 Effect of Diet and Disease on Animal Characteristics

Animal Group	Parameter						
	Body Weight*	Stomach Weight*	Intestine Weight$^{\phi}$	Intestine Weight / Body Weight$^{\sim}$	Intestine Length$^{\lambda}$	Colon Weight$^{\gamma}$	Colon Weight / Body Weight$^{\beta}$
CAS BBdp	84.7 ± 2.5	0.75 ± 0.04	1.03 ± 0.03	1.22 ± 0.06	91.2 ± 1.4^{b}	0.38 ± 0.01^{b}	0.45 ± 0.02
CAS BBn	76.6 ± 4.2^{b}	0.70 ± 0.04^{b}	1.07 ± 0.04^{ab}	1.39 ± 0.10^{b}	89.0 ± 1.6^{b}	0.38 ± 0.01	0.49 ± 0.03^{b}
CHOW BBdp	89.0 ± 3.0^{x}	0.69 ± 0.05^{x}	1.10 ± 0.04	1.24 ± 0.07^{x}	85.4 ± 1.7^{x,a}	0.37 ± 0.02^{b}	0.44 ± 0.02^{x}
CHOW BBn	105.5 ± 2.6^{y,a}	0.94 ± 0.04^{y,a}	1.02 ± 0.04^{b}	0.97 ± 0.07^{y,a}	98.4 ± 1.5^{y,a}	0.35 ± 0.01	0.34 ± 0.02^{y,a}
SOY BBdp	84.9 ± 2.3^{x}	0.75 ± 0.04	1.02 ± 0.03^{x}	1.21 ± 0.06^{x}	94.9 ± 1.3^{c}	0.43 ± 0.01^{x,a}	0.51 ± 0.02^{b}
SOY BBn	74.0 ± 2.6^{y,b}	0.70 ± 0.04^{b}	1.17 ± 0.03^{y,a}	1.60 ± 0.06^{y,b}	97 ± 1.4^{a}	0.38 ± 0.01^{y}	0.52 ± 0.02

Values are mean ± SEM (n=6-8 rats / treatment). Superscripts 'x' and 'y' represent a significant difference ($p<0.05$) between diseases. Superscripts 'a' to 'c' represent a significant difference ($p<0.05$) between diets within a disease.

* Significance for Body Weight (Diet $p<0.0001$; Disease NS; Diet*Disease $p<0.0001$)

* Significance for Stomach Weight (Diet $p<0.05$; Disease NS; Diet*Disease $p<0.001$)

$^{\phi}$ Significance for Intestine Weight (Diet NS; Disease NS; Diet*Disease $p<0.01$)

$^{\sim}$ Significance for Intestine Weight / Body Weight (Diet $p<0.004$; Disease NS; Diet*Disease $p<0.0001$)

[δ] Significance for Intestine Length (Diet $p<0.001$; Disease $p<0.001$; Diet*Disease $p<0.001$)

[γ] Significance for Colon Weight (Diet $p>0.001$; Disease $p<0.05$; Diet*Disease NS)

[β] Significance for Colon Weight / Body Weight (Diet $p<0.0001$; Disease NS; Diet*Disease $p<0.02$)

TABLE 6.3 Effect of Diet and Disease on Absolute Glucose Transporter mRNA Abundance

	Glucose Transporter	
Treatment Group	**SGLT-1 mRNA***	**GLUT5 mRNA***
CAS BBdp	9.99 ± 0.51	9.40 ± 0.86
CAS BBn	10.82 ± 0.58^{b}	11.37 ± 0.98^{b}
CHOW BBdp	8.97 ± 0.58^{x}	7.75 ± 0.98^{x}
CHOW BBn	13.31 ± 0.54^{y,a}	15.34 ± 0.92^{y,a}
SOY BBdp	9.42 ± 0.52^{x}	9.07 ± 0.87
SOY BBn	7.99 ± 0.54^{y,c}	7.52 ± 0.92^{c}

Values are mean ± SEM (n=6-8 rats / treatment). Values are densitometer units. Superscripts 'x' and 'y' represent a significant difference ($p<0.05$) between diseases. Superscripts 'a' to 'c' represent a significant difference ($p<0.05$) between diets within a disease.

* Significance for SGLT-1 mRNA (Diet $p<0.0002$; Disease $p<0.001$; Diet*Disease $p<0.0001$)

* Significance for GLUT5 mRNA (Diet $p<0.003$; Disease $p<0.001$; Diet*Disease $p<0.0001$)

TABLE 6.4 Effect of Diet and Disease on Total Potential Glucose Transporter mRNA Abundance in Total Jejunal RNA

	Glucose Transporter	
Treatment Group	**SGLT-1 mRNA***	**GLUT5 mRNA***
CAS BBdp	6.94 ± 0.63^{b}	6.58 ± 0.81
CAS BBn	8.00 ± 0.72^{b}	8.38 ± 0.92^{b}
CHOW BBdp	$8.70 \pm 0.72^{x,a}$	7.59 ± 0.92^{x}
CHOW BBn	$11.75 \pm 0.67^{y,a}$	$13.38 \pm 0.86^{y,a}$
SOY BBdp	7.37 ± 0.64^{ab}	7.09 ± 0.81
SOY BBn	8.00 ± 0.67^{b}	7.54 ± 0.86^{b}

Values are mean ± SEM (n=6-8 rats / treatment). Values are densitometer units. Superscripts 'x' and 'y' represent a significant difference ($p<0.05$) between diseases. Superscripts 'a' to 'c' represent a significant difference ($p<0.05$) between diets within a disease.

* Significance for SGLT-1 mRNA (Diet $p<0.0003$; Disease $p<0.006$; Diet*Disease NS)

* Significance for GLUT5 mRNA (Diet $p<0.001$; Disease $p<0.0005$; Diet*Disease $p<0.01$)

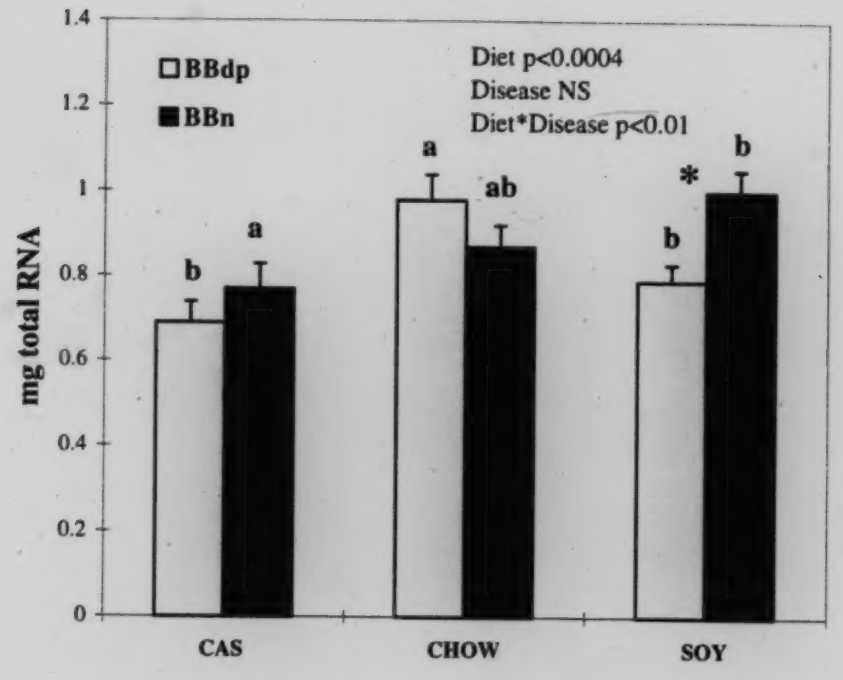

Figure 6.1 Effect of diet and disease on total jejunal RNA content in BB rats. Values were obtained by multiplying jejunal RNA concentration (mg/g) by total jejunal weight (g). Values are mean ± SEM (minimum n=7 rats / treatment). Significant effects are expressed in the figure.Superscripts 'a' and 'b' represent a significant difference between diets within a disease group ($p<0.05$).

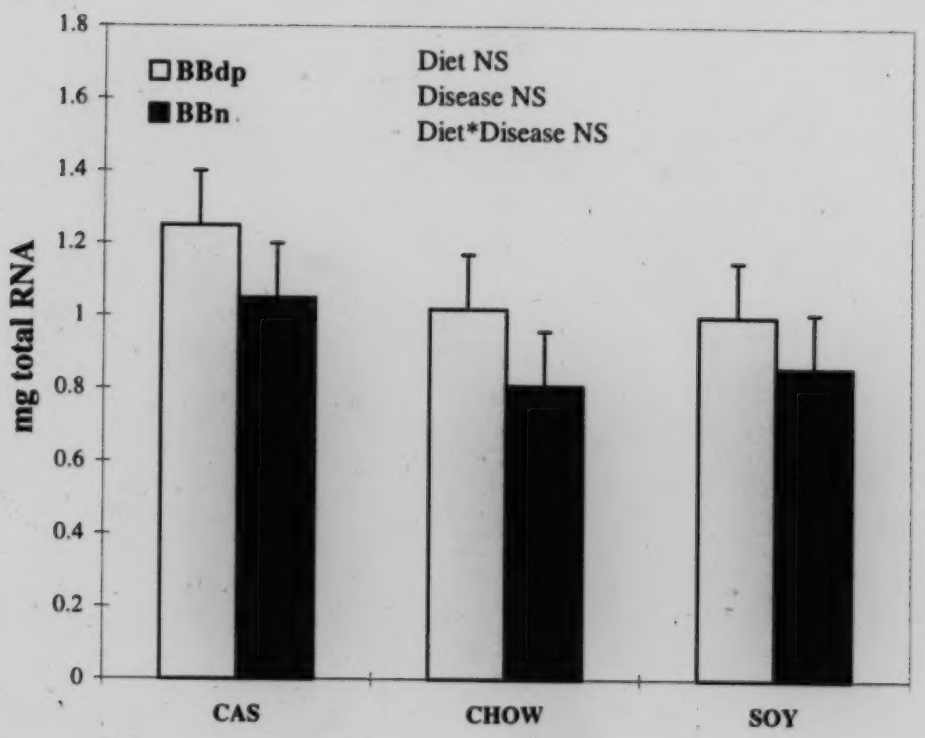

Figure 6.2 Effect of diet and disease on total colonic RNA content in BB rats. Values were obtained by multiplying colonic RNA concentration (mg/g) by total colonic weight (g). Values are mean ± SEM (minimum n=7 rats / treatment). Significant effects are expressed in the figure.

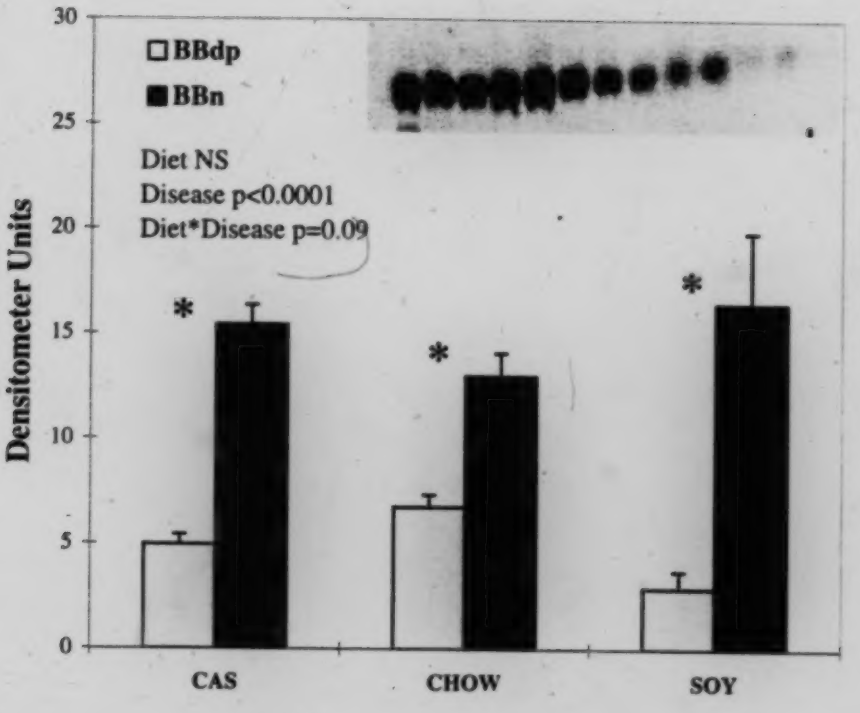

Figure 6.3 Effect of diet and disease on colonic proglucagon mRNA expression in BB rats. Values are mean ± SEM (n=4 rats / treatment). Significant effects are expressed in the figure. *Significant difference between BBdp and BBn ($p<0.05$). Inset of autoradiograph. Each lane was loaded with 15 ug of total RNA. From left to right in duplicate: BBn CAS, BBn CHOW, BBn SOY, BBdp CAS, BBdp CHOW, BBdp SOY.

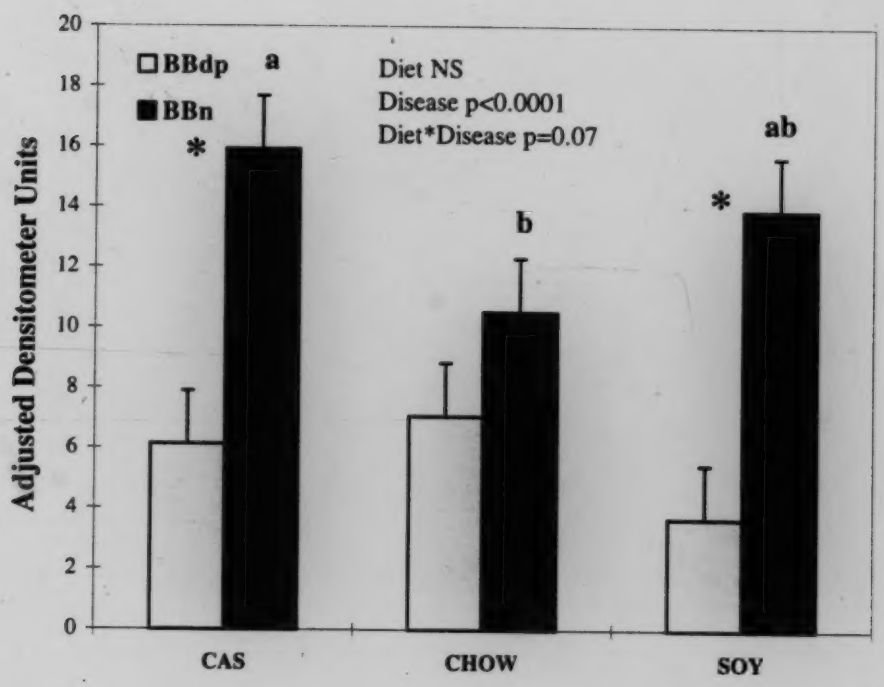

Figure 6.4 Effect of diet and disease on total potential colonic proglucagon mRNA abundance in total colonic RNA in BB rats.

Values are mean ± SEM (n=4 rats / treatment). Significant effects are expressed in the figure. Superscripts 'a' and 'b' represent a significant difference between diets within a disease group ($p<0.05$). * Significant difference between BBdp and BBn ($p<0.05$).

CHAPTER 7

INHIBITION OF INSULIN-STIMULATED GLUCOSE UPTAKE BY EPITROCHLEARIS MUSCLE IN VITRO BY GLP-1(7-36)AMIDE IN RATS

INTRODUCTION

The ability of gastrointestinal hormones to modulate the disposition of absorbed nutrients is supported by the increase in insulin secretion seen after the ingestion of oral nutrients or the incretin effect (McIntyre et al, 1964; Creutzfelt & Ebert, 1985). Glucagon-like peptide-1(7-36)amide seems to be primarily responsible for a large portion of the incretin effect (Wang et al, 1995). This peptide is derived from the proglucagon precursor and is produced in ileal and colonic mucosa (Kreymann et al, 1988; Orskov et al, 1989; Mojsov et al, 1990). Both GLP-1(7-36)amide and its nonamidated form (GLP-1(7-37)) are potent glucose-dependent insulintropins (Kreymann et al, 1987; Weir et al, 1989; Gutniak et al, 1992) The ability of GLP-1 to stimulate insulin secretion and lower postprandial hyperglycemia makes it a potentially useful therapeutic agent for the treatment of non-insulin dependent diabetes mellitus (NIDDM) (Gutniak et al, 1992; Nathan et al, 1992).[1]

The action of GLP-1(7-36)amide in extrapancreatic tissues has not been characterized to the same extent as in the pancreas. GLP-1 receptors, of the pancreatic type have recently been localized to the brain (Shimizu et al, 1987), lung (Kanse et al, 1988), gastric glands

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[1] A version of this chapter has been submitted for publication to Metabolism.

[2] This work was presented at Experimental Biology 96, April 1996, Washington, DC and published in part in abstract form [Reimer RA, Baracos VE, McBurney MI, Effect of dietary fiber and GLP-1 on uptake of glucose in skeletal muscle. FABEB Journal 10:A3011]. This work was also presented at Canadian Federation of Biological Societies, June 1996, London, Ontario and published in part in abstract form [Reimer RA, Baracos VE, McBurney MI, GLP-1 inhibits glucose uptake in skeletal muscle from rats in vitro. CFBS 39:A001].

of the stomach (Uttenthal & Blazquez, 1990), pancreas, kidney and the small and large intestine (Campos et al, 1994). GLP-1 has also been shown to bind with high affinity to isolated rat adipocytes, activate cAMP production (Valverde et al, 1993) and stimulate lipogenesis (Oben et al, 1991) or lipolysis (Ruiz-Grande et al, 1992). This effect, however, may not be mediated by the pancreatic type receptor. Sensitive methods of RT-PCR and ribonuclease protection have shown that the pancreatic type receptor transcript is absent in adipose, liver and skeletal muscle (Bullock et al, 1996).

Regardless of receptor type, the binding of GLP-1 in the key glucose utilizing non-islet tissues of muscle, adipose tissue and liver (Wheeler et al, 1993; Egan et al, 1994) make it plausible that GLP-1 has direct effects on glucose uptake and /or production. Skeletal muscle is a major glucose utilizing organ and the presence of GLP-1 receptors on this tissue suggests that GLP-1 may act as an important regulator of glucose metabolism (Delgado et al, 1995). Reports on the actions of GLP-1 at the muscle are yet controversial with Villanueva-Penacarillo et al (1994) describing potent glycogenic effects while Furnsinn et al (1995) were unable to reproduce those findings.

In order to further characterize the physiological actions of GLP-1 on muscle we investigated the effect of GLP-1(7-36)amide on non-insulin and insulin-stimulated glucose uptake in skeletal muscle. In addition, the recent discovery of the GLP-1 antagonist, exendin 9-39 (Goke et al, 1993) was used to confirm the specific actions of GLP-1 on glucose uptake and broaden our understanding on the role of GLP-1 in muscle and whole body glucose homeostasis.

MATERIALS AND METHODS

Animals Female Sprague-Dawley rats (150-180g) were obtained from the University of Alberta Health Sciences colony and housed in a temperature and humidity controlled room with a 12hr light/dark cycle. Animals were allowed free access to commercial laboratory chow (Rodent Laboratory diet PMI #5001, PMI Feeds Inc, St. Louis MO) and

water for ad libitum consumption. Animals were killed by cervical dislocation after an overnight fast. Epitrochlearis muscles were dissected intact for incubation.

Chemicals GLP-1(7-36) amide and exendin 9-39 were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Peptides were dissolved in distilled deionized water and stored in aliquots at -70 C. Immediately before use the solution was thawed and dissolved in Krebs Ringer bicarbonate buffer (KRB) (pH 7.4). All other chemicals used in preparation of the incubation media were purchased from Sigma Chemicals (St. Louis, MO) and BDH (Toronto, ON, Canada). 3-O-[^{3}H]methyl-glucose was purchased from New England Nuclear Corp. (Boston, MA; 300 uCi/mmol). L-[1-^{14}C]glucose was purchased from Amersham Life Sciences (Oakville, ON, Canada; 58.0 mCi/mmol).

Glucose Transport in Muscle Glucose transport was measured according to Liu et al (1995) with minor modifications. In all experiments, muscles were pre-incubated for 45 minutes followed by a second incubation with labelled 3-O-[^{3}H]methyl-glucose 30 minutes. Muscles were preincubated for 45 minutes at 29 C in 3 ml of Krebs Ringer bicarbonate buffer (KRB) (NaCl, 119 mM; KCl, 4.82 mM; $MgSO_4$, 1.25 mM; NaH_2PO_4, 1.24 mM; $NaHCO_3$, 25 mM; N-2-hydroxyethylpeperazine-N-2-ethane sulfonic acid (HEPES) (pH 7.4), 2.0 mM; $CaCl_2$, 1.0 mM), supplemented with 8 mM D-glucose, 32 mM mannitol and 0.1% BSA and saturated with 95% O_2-5% CO_2 gas mixture. For the various experiments muscles were pre-incubated with or without different concentrations of GLP-1 and/or exendin 9-39. Muscles were transferred into fresh media containing 8 mM 3-O-[^{3}H] methyl glucose and ^{14}C-L-glucose, 32 mM mannitol and 0.1% BSA. The presence or absence of GLP-1(7-36)amide and exendin 9-39 in various concentrations varied according to experiment. According to defined experimental conditions, 0.8 nM insulin (a half-maximally stimulating dose as defined previously,(Liu et al, 1995)) was also present or absent from the incubation period with labelled glucose. Incubation time was 30 minutes at 29 C depending on the experimental conditions established. Muscles were then removed from the incubation media and washed three times each for 10

minutes with ice cold KRB to remove labelled glucose from the extracellular space (Poggi et al, 1979). Muscles were blotted, solubilized with Soluene 350 (Packard, Downers Grove, Il) and counted in a mixture of 5 ml Hionic-Fluor (Packard) and 10 ml EcoLite™ Scintillation Cocktail (ICN, Mississauga, Canada). Previous studies have shown that an accurate correction for nonspecifically bound and passively diffused 3-O-[^{3}H] methyl glucose is the subtraction of the amount of ^{14}C-L-glucose remaining in the muscle after washing from the amount of 3-O-[^{3}H] methyl glucose (Liu et al, 1995).

Statistical Analysis Data are given as mean $\pm$ SEM. Differences between treatments were determined using the one-way ANOVA model in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Multiple comparisons were made using Duncan's Multiple Range test. Statistical significance is defined as $p \leq 0.05$.

RESULTS

The addition of either 50 pM or 300 pM GLP-1 to the labelled incubation media did not alter glucose uptake under basal or insulin stimulated conditions (**Figure 7.1**). The normal increment in glucose uptake seen under insulin stimulated states was unaltered by the various GLP-1 treatments (**Figure 7.1**). Exposure of the muscles to GLP-1 strictly in the 30 minute incubation period did not affect non-insulin or insulin stimulated glucose uptake.

Addition of 50 pM or 300 pM GLP-1 to the 45 minute pre-incubation period in addition to the 30 minute incubation period did not affect the uptake of 3-O-[^{3}H] methyl glucose under non-insulin stimulated conditions (**Figure 7.2**).

Lengthening the duration of exposure to GLP-1 had a profound effect on insulin stimulated glucose uptake. Exposure of the muscles to GLP-1 in the 45 minute preincubation period prior to the 30 minute incubation period resulted in a significant inhibition of insulin stimulated glucose uptake in a concentration dependent fashion

(Figure 7.3). The level of insulin stimulated glucose uptake was significantly lower with the addition of 300 pM GLP-1. Glucose uptake in the presence of 50 pM GLP-1 was intermediate between 0 pM and 300 pM GLP-1 concentrations.

Using exendin 9-39 amide in the presence of insulin at a concentration 10 times that of GLP-1 did not block the inhibitory effect of GLP-1 on glucose uptake **(Figure 7.4)**. Incubation of muscles with exendin alone resulted in glucose uptake that was not significantly different from 0.8 nM insulin stimulated uptake. At concentrations 1000 fold that of GLP-1, exendin did not block the effects of GLP-1 on insulin stimulated glucose uptake **(Figure 7.5)**. In addition, at this higher concentration, exendin 9-39 amide decreased glucose uptake to the same extent as 300 pM GLP-1.

DISCUSSION

Glucagon-like peptide-1 has potent actions on insulin secretion and is thought to have potential as a therapeutic agent for the treatment of non-insulin dependent diabetes mellitus (Orskov, 1992; Fehmann & Habener, 1992). Previous reports of the antidiabetogenic actions of GLP-1 and the recent demonstration the GLP-1 binds to skeletal muscle suggests that GLP-1 may potentiate its pancreatic insulinogenic actions and improve glucose regulation by enhancing insulin stimulated glucose uptake in the epitrochlearis muscle.

We were surprised to find that prolonged exposure of epitrochlearis muscle to 300 pM GLP-1 significantly impairs normal insulin stimulated glucose uptake. Only recently have investigators begun to examine the effects of long term administration of GLP-1 *in vivo*. These reports stem from recent experiments showing that the GLP-1 receptor in rat insulinoma cells is subject to rapid homologous desensitization (Fehmann & Habener, 1991; Gromada et al, 1996). High concentrations of GLP-1 have also been shown to inhibit cAMP production and insulin release (Gefel et al, 1990; Drucker et al, 1987; Goke

et al, 1988). The use of isolated rat pancreatic islets as a more physiological model demonstrates the loss of insulinotropic effect of GLP-1 in long term incubations as well (Gronau et al, 1995). These reports suggest that normal islets may undergo homologous desensitization during prolonged exposure to GLP-1. Although no reports exist to the desensitization of GLP-1 receptors on muscle, the reduction of insulin stimulated glucose uptake in muscle exposed to longer periods of GLP-1 in this study suggest that the receptor at this site may also be subject to desensitization.

In an effort to elucidate the effect of GLP-1 on glucose uptake prior to receptor desensitization we incubated muscles for 5 minutes in the presence or absence of GLP-1. We were unable to consistently obtain levels of glucose uptake indicating insulin stimulated conditions and speculate that as with maximally stimulated (20 nM insulin) glucose uptake (Liu et al, 1995), uptake at 0.8 nM may not be linear. This phenomenon theoretically limits our ability to confidently measure the acute effects of GLP-1 on glucose uptake with the present *in vitro* model. The effect of GLP-1 on muscle glucose uptake with brief (5 min) exposure to GLP-1, therefore, remains to be elucidated.

Studies indicate that two peptides isolated from the venom of the lizard *Heloderma suspectum*, exendin-4 and exendin 9-39 are an avid agonist and antagonist respectively of the pancreatic type GLP-1 receptor (Goke et al, 1993; Raufman et al, 1992; Thorens et al, 1993). The discovery of exendin 9-39 has been an important tool in studying the specific contribution of GLP-1 to the incretin effect. In this study we set out to demonstrate that the inhibition of glucose uptake was a specific GLP-1 receptor related phenomenon by using the antagonist exendin 9-39 amide.

In the pancreas previous *in vitro* experiments have shown exendin 9-39 amide needs to be applied in at least a 10 fold excess to reduce GLP-1 effects on insulin secretion (Goke et al, 1993). In muscle, neither 10 fold or 1000 fold exendin concentrations were able to block GLP-1 actions on glucose uptake. In fact 1000 fold concentration of exendin 9-39

alone inhibits glucose uptake to the same extent as 300 pM GLP-1. Delgado et al (1995) have demonstrated GLP-1(7-36)amide-specific binding in skeletal muscle plasma membrane. GLP-1 did not modify basal adenylate cyclase activity in muscle plasma membranes. In addition, previous findings of a glycogenic effect of GLP-1 on soleus muscle occurred without an increase in cAMP content (Villaneuva-Penacarrillo et al, 1994). Taken together with these reports our data suggests that GLP-1 mediated effects at muscle may occur via a receptor different from that characterized for pancreatic B cells. Indeed, attempts to locate mRNA encoding the pancreatic form of the GLP-1 receptor have not detected the message in adipose, liver or skeletal muscle (Bullock et al, 1996).

To date, two conflicting reports exist as to the actions of GLP-1 in muscle. Villanueva-Penacarrillo et al (1994) demonstrated a potent glycogenic effect of GLP-1 in rat skeletal muscle. Furnsinn et al (1995) were unable to reproduce any glycogenic effects of GLP-1 in a similar set of experiments. Our findings now suggest that prolonged exposure to GLP-1 may reduce insulin stimulated glucose uptake and ultimately insulin sensitivity. In both studies examining the glycogenic actions of GLP-1, muscles were incubated in GLP-1 or insulin exclusively. Our study in attempting to mimic the physiological environment of the muscle examined the effects of GLP-1 both in the presence and absence of insulin. In addition we have used a higher glucose concentration (8 mM versus 5 mM) in our incubation media and fasted animals prior to the experiment. Villanueva-Penacarrillo et al (1994) demonstrated a glycogenic GLP-1 effect in fed rats whereas Furnsinn et al (1995) used fasted rats and was unable to demonstrated similar findings. We propose that these key variables may help explain the divergent results of GLP-1 actions in muscle thus far. The potent actions of GLP-1 on insulin-stimulated glucose uptake in muscles exposed to GLP-1 for prolonged periods suggests that the interaction between insulin and GLP-1 is an important factor in the characterization of the actions of GLP-1.

In summary, the present studies demonstrate that insulin stimulated glucose uptake in

skeletal muscle is decreased with prolonged exposure to high concentrations of GLP-1. Exendin 9-39 an analogue of GLP-1 and antagonist of the pancreatic GLP-1 receptor is unable to block GLP-1 effects and actually results in decreased glucose uptake independent of GLP-1 at high concentrations. The effect of acute exposure to GLP-1 remains to be elucidated. The importance of skeletal muscle as a major glucose utilizing tissue in the body warrants the further examination of the potentially deleterious effects of long term administration of GLP-1.

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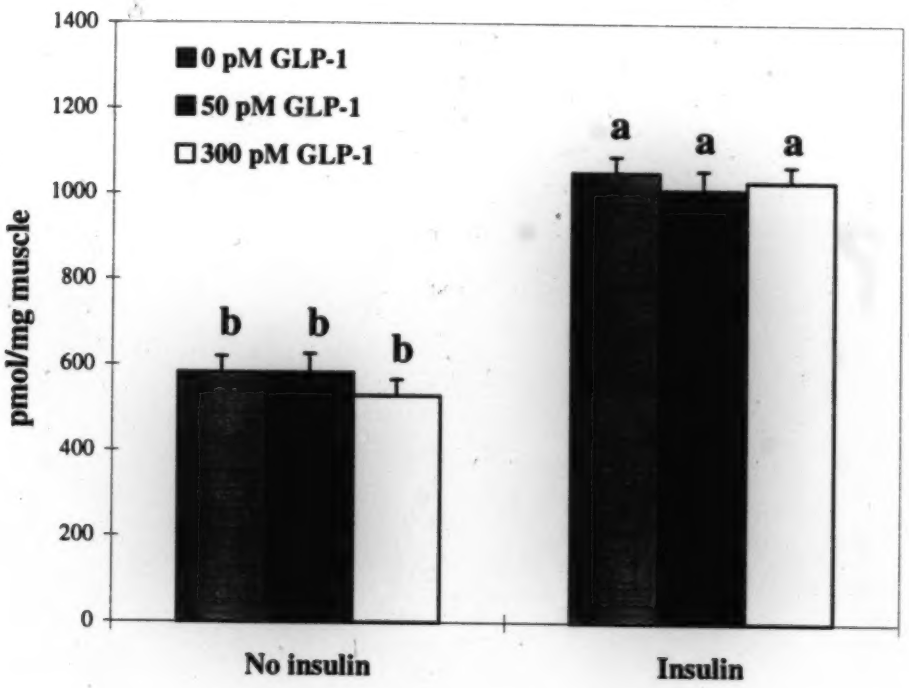

Figure 7.1 Effect of short-term exposure to different concentrations of GLP-1(7-36) amide on glucose uptake in epitrochlearis muscle. GLP-1 was not present in the 45 minute preincubation period. Insulin was added to half of the tubes from each treatment during the incubation period to obtain insulin stimulated values. Values are mean ± SEM (n=7 rats / treatment). Values with different letters are significantly different ($p<0.05$).

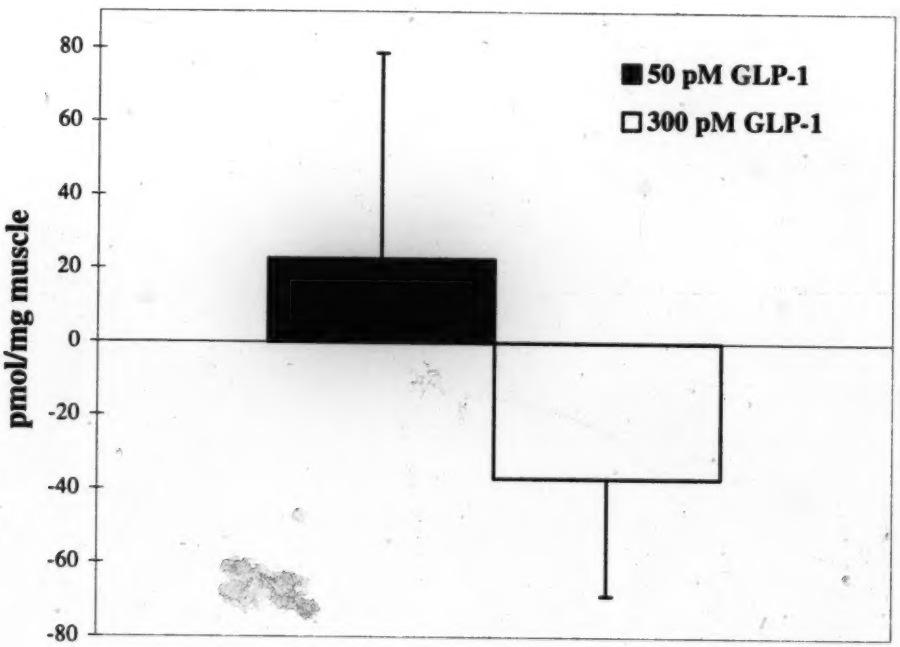

Figure 7.2 Effect of short-term exposure to different concentrations of GLP-1(7-36) amide on uptake of glucose in epitrochlearis muscle under non-insulin stimulated conditions. GLP-1(7-36) amide was present in the 45 minute pre-incubation period. Values represent the difference between glucose uptake in the presence of GLP-1 minus glucose uptake under basal (0 pM GLP-1) conditions. Values are mean ± SEM (n = 8 rats / treatment).

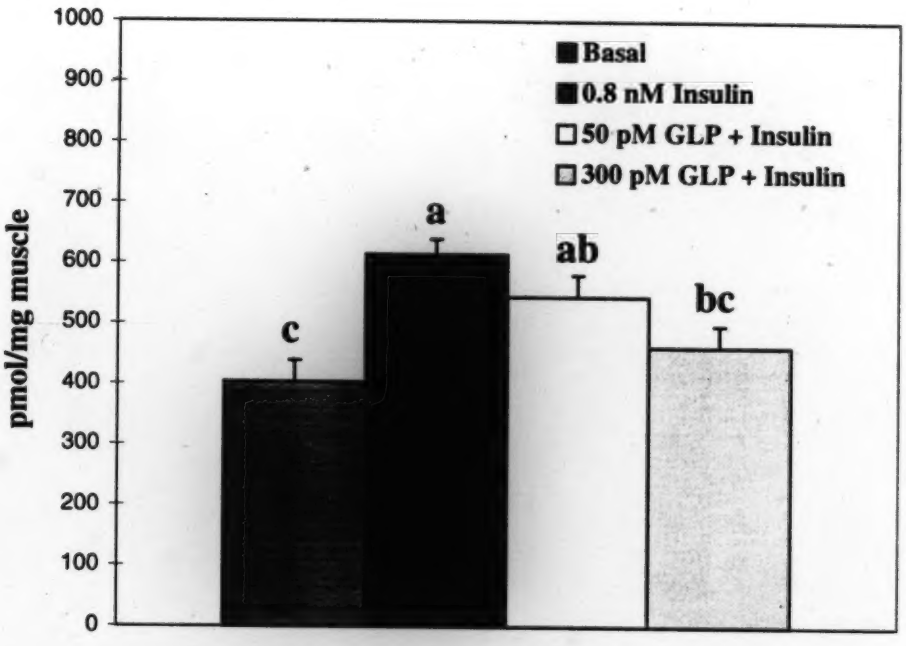

Figure 7.3 Effect of prolonged exposure to different concentrations of GLP-1(7-36) amide on insulin-stimulated glucose uptake in epitrochlearis muscle. GLP-1 was present in the 45 min preincubation period. Insulin was added to half of the tubes from each treatment to obtain insulin stimulated values from the incubation period. Values are mean ± SEM (n=8 rats / treatment). Values with different letters are significantly different ($p<0.05$).

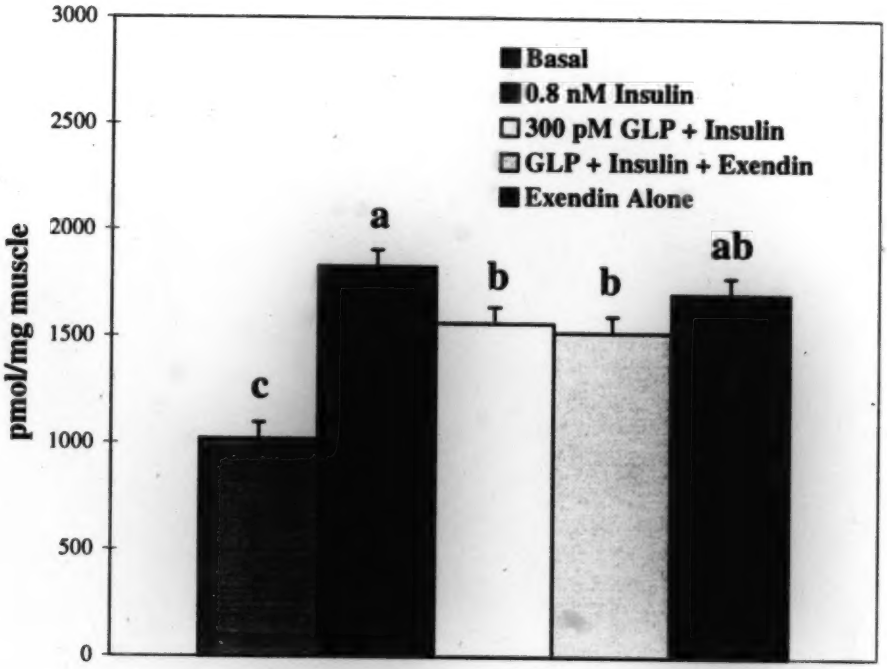

Figure 7.4 Effect of exendin 9-39 amide (at 10X the concentration of GLP-1) on glucose uptake in epitrochlearis muscle. GLP-1 and/or exendin were present in the 45 min preincubation period. Insulin was added to all treatments except 'basal' during the incubation period. Values are mean ± SEM (n=9 rats / treatment). Values with different letters are significantly different ($p<0.05$).

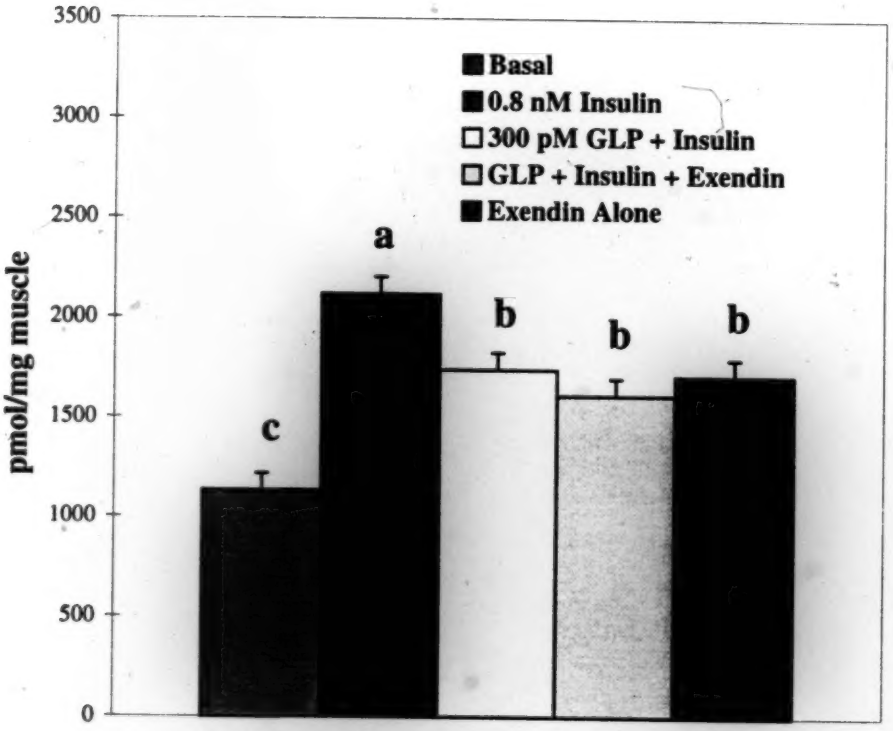

Figure 7.5 Effect of exendin 9-39 amide (at 1000X the concentration of GLP-1) on the uptake of glucose in epitrochlearis muscle. GLP-1 and/or exendin were present in the 45 min preincubation period. Insulin was added to all treatments except 'basal' during the incubation period. Values are mean ± SEM (n=9 rats / treatment). Values with different letters are significantly different ($p<0.05$).

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

Adaptation of the intestine in response to luminal nutrients is a complex physiological process that is not completely understood. Intestinal delivery of nutrients and hormones to the systemic circulation may significantly affect metabolism and the risk and/or management of chronic diseases. Questions specific to the molecular mechanisms governing morphological and functional adaptation of the intestine to these nutrients remain largely unanswered. Dietary fiber appears to play a role in glucose homeostasis by altering the balance between glucose absorption and glucose utilization. My goal was to examine the effect of dietary fiber on the synthesis of gut glucose transporters and the insulinotropic gastrointestinal hormone, proglucagon.

EXPERIMENTAL STRATEGIES

The objectives of this research were:

1) to determine the role dietary fiber and proglucagon-like peptides play in glucose homeostasis in normal and diabetic adult animals;
2) to characterize the effect of diet on proglucagon and glucose transporter gene expression in the gastrointestinal tract of 7-30 day old diabetes prone and normal BB rats;
3) to characterize the actions of the intestinal hormone, GLP-1, on skeletal muscle glucose uptake.

Several experiments were performed to achieve these objectives and an explanation for some of the methods and strategies employed is provided.

Diets

Three diets differing in quantity of fiber and fiber type were used in these studies. Previous work in our laboratory had demonstrated effects of a 30% (wt/wt) fiber diet on colonocyte metabolism. The fiber source employed was moderately fermentable and composed of a 5:1 mixture of Fibrad™ and cellulose. Using this diet, the effects of a high fiber versus elemental diet on intestinal adaptation were examined in relation to proglucagon-derived peptides and glucose homeostasis. Having established that this high (30%) fiber diet upregulates proglucagon mRNA and increases GLP-1 and insulin secretion in Chapter 2 the effects of fiber fermentability per se were examined at a more physiological level (5% wt/wt). Many studies have used cellulose fiber as a standard non-fermentable fiber source at this level (Gee et al, 1996; Southon et al, 1987). Rhubarb fiber was chosen as the comparison because of its fermentability compared to cellulose (6.5 mmol/g vs 2.5 mmol/g organic matter, respectively).

Standard laboratory chow was used in the initial study characterizing intestinal ontogeny in weanling BBn and BBdp animals. Weaning to chow diets produces the highest incidence of diabetes in BBdp rats compared to semi-purified diets (Scott et al, 1988; Hoorfar et al, 1993). Although the role of protein in weaning diets has been extensively studied (Scott et al, 1988), the importance of carbohydrate content in these diets remains to be elucidated. Standard laboratory chow contains nearly 50% (wt/wt) carbohydrate compared to the semi-purified diets used in these studies which contain only 27% (wt/wt) carbohydrate. The effects of a higher carbohydrate load on subsequent adaptation by the developing intestine warrant examination. Thus, diets known to influence the incidence and onset of diabetes were fed to examine their influence on intestinal development.

***In Vitro* Muscle Studies**

Skeletal muscle is a major glucose utilizing tissue of the body. The metabolism of carbohydrate in skeletal muscle is sensitive to nutritional and hormonal factors. The recent demonstration that GLP-1, a potent insulin secretagogue, binds to skeletal muscle in rats suggests that this hormone may regulate glucose disposal at this site (Delgado et al, 1995). The actions of GLP-1 in muscle, however, remain largely unknown. The uptake of labelled ^{3}H-3-O-methylglucose into muscle was used to determine the effects of GLP-1 on the first step in muscle glucose metabolism, glucose transport. Epitrochlearis muscle was chosen for these studies because it is less than 0.2 mm in thickness and therefore allows for adequate oxygenation of all cells and ensures viability during the experimental period (Nesher et al, 1980).

Use of RNase Protection Assays, Northern Blot Analysis and Radioimmunoassay

Measurement of mRNA provides insight into factors which modulate expression of genes, the first stage of functional change. In these studies the effects of dietary fiber and ontogenic development on specific mRNA were examined. Northern blot analysis is a technique used in molecular biology to determine the size and expression pattern of specific mRNA's. The RNase protection assay also measures specific mRNA's but is several times more sensitive than northern analysis because hybridization of total RNA and the labelled probe occurs in solution as opposed to on a nitrocellulose support matrix. RNase protection assay was used in the first study as a sensitive method by which to determine any effect fiber may have on proglucagon mRNA. Subsequent work confirmed that northern blot analysis was of adequate sensitivity to detect the mRNA's of interest and was therefore applied for the duration of studies.

Messenger RNA templates are translated into the polypeptide chains of proteins. Changes in mRNA are physiologically meaningful if concomitant changes also occur in the biologically active peptides which they produce. For this reason radioimmunoassays were used for the measurement of plasma GLP-1. Radioimmunoassay kits obtained from

Peninsula Laboratories were used for GLP-1(7-37) and GLP-1(7-36) amide measurement. More recently a GLP-1(7-36) amide antibody was generously donated by Dr. Chris McIntosh of the University of British Columbia for radioimmunoassay.

DIETARY FIBER IMPROVES GLUCOSE HOMEOSTASIS VIA CHANGES IN GASTROINTESTINAL HORMONE EXPRESSION

> **Hypothesis 1:** Consumption of diets high in fermentable fiber upregulates proglucagon gene expression and secretion of GLP-1 and insulin in normal and diabetic adult rats.

In normal rats, supplementing an elemental diet with 30% fiber (wt/wt) increased ileal, cecal and colonic mass and increased total colonic RNA. Ileal proglucagon mRNA and the secretion of GLP-1, insulin and c-peptide were also elevated after oral glucose with long term ingestion of the 30% fiber diet. Although intestinal mass was not increased, the more fermentable rhubarb fiber increased ileal proglucagon mRNA and plasma concentrations of c-peptide 30 minutes after an oral glucose load. Tappenden et al (1996) demonstrated that supplementing total parenteral nutrition with SCFA upregulated proglucagon mRNA after intestinal surgery and suggests that this important intestinal fuel may regulate changes observed with dietary fiber. Future investigation using in situ hybridization should elucidate whether the number of proglucagon producing L-cells increases with the ingestion of dietary fiber or, conversely, whether the number of L-cells remains static but an enhanced abundance of message per L-cell results with the ingestion of fiber. Thus, observations from Chapters Two and Three suggest that diet, specifically dietary fiber, can be modulated to "engineer" the gene profile of the gastrointestinal tract. Moreover, the beneficial effects of fiber on glycemia and insulin secretion seem to be mediated via changes in expression of gastrointestinal hormones in normal animals.

Diabetic animals, however, respond differently to dietary fiber than normal animals. Proglucagon mRNA increased in the ileum and colon of diabetic versus normal animals. SGLT-1 and GLUT2 mRNA increased in diabetics versus normals as well. Although pair-feeding tends to 'normalize' some parameters, a diabetes specific effect on gut proglucagon mRNA is still observed, as has been reported by others for glucose transport (Fedorak et al, 1990; Burant et al, 1994).

The ingestion of rhubarb fiber did not upregulate intestinal proglucagon mRNA in diabetic animals. The diabetic rat intestine is known to be hypertrophic (Pillion et al, 1983) and may be incapable of mounting a hypertrophic response to fermentable fibers as observed with normal animals ingesting high fiber diets long-term. Proglucagon mRNA was much greater on cellulose in diabetics whereas the differences between diabetics and normals was smaller on rhubarb. Food intake or amount of food ingested was an important factor regulating plasma glucose, insulin and c-peptide as well as the abundance of SGLT-1 and GLUT2. Proglucagon mRNA and plasma GLP-1(7-36)amide were altered by the ingestion of fermentable fiber but not food intake per se. These observations suggest that proglucagon-derived peptides respond to fermentation end-products, whereas SGLT-1 and GLUT2 mRNA and plasma glucose and insulin also respond to the delivery of nutrients into the small intestine per se. Further investigations are needed to determine mechanisms by which diet, diabetes and proglucagon-derived peptides interact to influence glucose homeostasis.

It is clear that different metabolic states (normal, streptozotocin-induced diabetes and autoimmune diabetes) respond differently to various fibers. Future studies will be needed to define the optimum level and type of fiber in diets to optimize the balance between glucose input (intestinal absorption) and glucose disposal (insulin secretion) in adult diabetics.

DIETARY FIBER ENHANCES DISEASE PROGRESSION IN THE YOUNG BB DIABETES PRONE RAT

Hypothesis 2: Consumption of diets high in fermentable fiber between 21 and 30 days of age increase proglucagon and glucose transporter mRNA abundance in BBn and BBdp animals.

Characterization of the intestine is similar in BBn and BBdp rats until 14 days of age when BBdp animals begin to exhibit greater intestinal mass as a proportion of their body weight. Exacerbating the increase in mass is the observed increase in glucose transporter mRNA in the BBdp animals. Alterations begin during the period associated with the first ingestion of dams diet at 14 days of age and become highly defined at the 'critical time' of weaning (21 days of age). Other studies have demonstrated that the protective effects of semi-purified diets are recognized only if the diet is introduced at least by the time of weaning (Scott & Marliss, 1991).

Endogenous insulin secretion is associated with insulitis and increased diabetes progression (Mordes et al, 1987). Because of its potent insulinotropic actions, GLP-1 may initiate or promote the autoimmune attack on the pancreatic beta cells. There was significantly reduced proglucagon mRNA abundance in BBdp versus BBn animals from 21 to 30 days of age. However, given the significantly higher amount of total RNA, BBdp animals actually have higher estimated total proglucagon mRNA from 21 to 30 days of age. This prediction is supported by the greater levels of plasma GLP-1 in BBdp versus BBn animals observed at 30 days of age.

BBdp animals fed chow had greater glucose transporter mRNA abundance at weaning (21d) compared to BBn animals who displayed peak levels at 30d. The recent discovery that GLP-2, another proglucagon-derived peptide, increases glucose uptake in the jejunum (Cheeseman & Tsang, 1996) suggests that this hormone may be involved in upregulation of glucose transporter mRNA in the small intestine. It appears that fiber

results in a double insult in terms of increasing requirements for insulin secretion. Both increased intestinal glucose absorption requiring enhanced insulin secretion for glucose disposal in tissues and an increase in the hormone (GLP-1) known to be a potent insulin secretagogue potentially heighten the autoimmune attack on the pancreas. The tendency for lower proglucagon mRNA abundance and the apparent peak of glucose transporter mRNA at 30d in BBdp fed casein and soy diets suggests that the reduced glucose entry rates and decreased insulinogenic stimulus might delay the onset of diabetes.

It has been suggested that activation of glucose transporters at the brush border membrane stimulates the condensation of actomysin associated with the tight junction and results in its contraction. The contraction opens the tight junction and permeability between cells increases (Madara et al, 1987). Glucose and other molecules may then cross the epithelium through the tight junction by solvent drag (Asitook et al, 1990). The possibility that the increased glucose transporter message in the BBdp is translated into an enhanced rate of glucose transport and a more 'leaky' gut may have implications for oral tolerance and immune response to foreign antigens absorbed via tight junctions. Implication of GLP-2 in not only altering tight junction integrity but the impact on increased nutrient availability and subsequent insulin secretion suggest that future studies should examine the role this hormone plays in intestinal adaptation seen in the weanling BBdp rat. IL-1, IL-10 and TNF-α have been implicated in the pathogenesis of autoimmune diabetes (Mueller et al, 1992). It is possible that diet alters the expression of these cytokines. K.A. Tappenden demonstrated that IL-1β increased with SCFA supplementation of TPN after massive small bowel resection (unpublished data). Perhaps alterations in gut associated cytokine production is involved in disease initiation and progression. The availability of cDNA's for these cytokines makes possible the future examination of these genes in the BB rat in response to chow and semi-purified diets. Further studies however examining changes in glucose transporter expression in response to casein and soy diets at time points earlier than 30d are needed.

CHARACTERIZATION OF THE ACTIONS OF GLP-1 ON SKELETAL MUSCLE

Hypothesis 3: GLP-1 stimulates insulin-stimulated glucose uptake by skeletal muscle *in vitro*.

GLP-1 may improve glucose homeostasis via increased insulin secretion (Holst, 1994). The presence of binding to three key glucose regulating tissues, liver, muscle and adipose suggested it may play a role in glucose utilization in extra-pancreatic tissues but prolonged exposure of muscle to GLP-1 inhibited insulin-stimulated glucose uptake in epitrochlearis muscle. Our findings are supported by reports that the pancreatic form of the GLP-1 receptor undergoes rapid homologous desensitization and more recently by reports of limited benefits of GLP-1 when infused long-term (Fehmann & Habener, 1991; Kawai & Ohashi, 1993). The fact that exendin 9-39, a homologue of GLP-1 inhibits glucose uptake as well lends further support to the speculation that skeletal muscle may not express the pancreatic form of the GLP-1 receptor. The potential use of GLP-1 as a therapeutic agent for NIDDM make further studies in the role of GLP-1 in whole body glucose homeostasis necessary.

In conclusion, this work demonstrated that fermentable fiber increases proglucagon synthesis. Diabetic animals have increased proglucagon mRNA and glucose absorption but fermentable fiber seems beneficial via increased GLP-1 versus glucose entry rate. Fermentable fiber in weanling diets may accelerate proglucagon expression and glucose absorption to increase risk of early-onset diabetes. Finally, prolonged exposure to GLP-1 may decrease glucose uptake by skeletal muscle. These studies have examined several molecular mechanisms governing intestinal adaptation in response to diet and specifically dietary fiber. Alterations in various markers of glucose absorption and disposal have been examined. Based on this work, additional experiments will be conducted which will ultimately enable nutrition experts to design diets, whether for infancy or adulthood, which optimize intestinal function and promote glucose homeostasis.

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